THE UNIVERSITY
of EDINBURGH

Protein secretion and encystation in *Acanthamoeba*

Alvaro de Obeso Fernández del Valle

THE UNIVERSITY *of* EDINBURGH

Doctor of Philosophy

The University of Edinburgh

2018

Abstract

Free-living amoebae (FLA) are protists of ubiquitous distribution characterised by their changing morphology and their crawling movements. They have no common phylogenetic origin but can be found in most protist evolutionary branches. *Acanthamoeba* is a common FLA that can be found worldwide and is capable of infecting humans. The main disease is a life altering infection of the cornea named *Acanthamoeba* keratitis. Additionally, *Acanthamoeba* has a close relationship to bacteria. *Acanthamoeba* feeds on bacteria. At the same time, some bacteria have adapted to survive inside *Acanthamoeba* and use it as transport or protection to increase survival. When conditions are adverse, *Acanthamoeba* is capable of differentiating into a protective cyst. This study had three objectives. First, isolate and identify new FLA and *Acanthamoeba* strains. Second, identify encystation factors of *Acanthamoeba*. Third, identify and characterise new potential antimicrobial proteins produced by *Acanthamoeba*. The isolation of environmental amoebae was performed, and several strains of *Acanthamoeba* were identified from previously known genotypes. Also, two new species of FLA were identified: *Allovahlkampfia minuta* and *Leptomyxa valladaresi*. The dynamics of encystment were studied in different strains of *Acanthamoeba*. RNAseq was used to study gene expression during differentiation and identify differentially expressed genes. We identified different encystment factors including at least two encystment related proteases. A new antimicrobial zymogram was developed that identified antimicrobial proteins being secreted by *Acanthamoeba*. A 33 kDa protease was found to be able to lyse bacteria. We created DNA constructs encoding the protease and a lysozyme from *Acanthamoeba* for heterologous expression. The genes were successfully cloned. However, bacteria were not able to produce the proteins most probably due to their antimicrobial characteristics. Further studies are required regarding encystment and antimicrobial factors identified. Such experiments should help elucidate critical factors of *Acanthamoeba*'s biology that could help treat several infections.

Lay summary

Free-living amoebae are microbes of ubiquitous distribution. Although they do not have a common evolutionary origin, all FLA are similar in their range of morphologies and locomotion. Some of these FLA are of particular importance as they are capable of producing infection. *Acanthamoeba* can produce a life-altering eye infection, among other less common diseases. In the environment, *Acanthamoeba* feeds on bacteria, and for this, it has developed different strategies to digest them. At the same time, some bacteria have adapted to be able to withstand *Acanthamoeba*, and survive and multiply inside of the amoeba. Bacteria that can survive inside *Acanthamoeba* can use it as a transport mechanism and as a haven from environmental challenges. Additionally, when conditions are adverse, *Acanthamoeba* can change its morphology and produce a protective cyst until conditions improve. This study had three objectives. First, isolate new amoebae. Second, identify critical factors in the production of the cyst by *Acanthamoeba*. Third, identify proteins used by *Acanthamoeba* to kill bacteria. Several *Acanthamoeba* organisms were isolated as well as two new species of FLA. In addition, different experiments to observe encystment dynamics were performed. From these, several factors for encystment were identified. Furthermore, two different proteins with potential to lyse bacteria were selected. One of the proteins was selected after the development of a new technique to observe bacterial lysis on a gel and the other protein through literature research. The genes for these proteins were isolated, but the proteins could not be produced in the laboratory. Although antimicrobial and encystment factors were identified, future studies are required to characterise them and have a deeper understanding of their functions and mechanisms. Such experiments should help elucidate critical factors of *Acanthamoeba*'s biology that could help treat several infections.

Own Work Declaration

I declare that the thesis has been composed by myself and that the work has not be submitted for any other degree or professional qualification. I confirm that the work submitted is my own, except where explicitly stated otherwise or where work which has formed part of jointly-authored publications has been included. My contribution to these works have been explicitly indicated below. I confirm that appropriate credit has been given within this thesis where reference has been made to the work of others.

Work presented in Chapter 5 and found in Appendix 1 was previously published in Experimental Parasitology as "*Leptomyxa valladaresi* n. sp. (Amoebozoa, Tubulinea, Leptomyxida), from Mount Teide, Tenerife, Spain" by Alvaro De Obeso Fernandez Del Valle, Jacob Lorenzo-Morales and Sutherland K. Maciver. This study was conceived by all of the authors. I carried out the molecular identification of the new organism.

Work presented in Chapter 5 and found in Appendix 1 was previously published in Acta Protozoologica as "*Allovahlkampfia minuta* nov. sp., (Acrasidae, Heterolobosea, Excavata) a New Soil Amoeba at the Boundary of the Acrasid Cellular Slime Moulds" by Alvaro De Obeso Fernandez Del Valle and Sutherland K. Maciver. This study was conceived by all of the authors. I carried out the isolation and molecular identification of the new organism.

Alvaro de Obeso Fernández del Valle

January 2018

Table of Figures

Table of Tables

Acknowledgements

The research was performed with the support of Mexico's National Council of Science and Technology (CONACYT) with the studentship 399135 and a University of Edinburgh College of Medicine and Veterinary Medicine PhD Studentship.

I would like to thank my supervisor Sutherland Maciver for all the help, patience and guidance provided during the PhD. I would also like to thank the other members of my PhD committee Mike Cousin and Kim Picozzi.

Finally, I would like to thank all the people that one way or another were instrumental during these almost 4 years of PhD, starting with my family, and also in no particular order: the people from HRB that were always willing to give a hand, members of the Mexican and Latin American Society, the CEJ, perkins, my flatmates Marisol and Alessandra, Selina for helping the thesis look good and all others that are probably too many to mention by name.

Abbreviations

AK	Acanthamoeba keratitis
ATSA	*Acanthamoeba*-specific amplimer S1
AX2	Axenic amoeba culture
Bp	Base pairs
CNS	Central nervous system
COI	Cytochrome oxidase subunit I
CPM	Counts per million
CSP21	Cyst specific protein 21 kDa
ddH_2O	Double distilled water
DE	Differentially expressed
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
FC	Fold change
FDR	False discovery rate
FLA	Free-living amoebae
GAE	Granulomatous amoebic encephalitis
GTSA.B1	Genotype-specific amplimer B1
ITS	Internal transcribed spacer
kDa.	Kilodaltons
LB	Lysogeny broth
LD	Legionnaires' disease
NEM	Neff's encystment media
NS	Neff's saline

OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PHMB	Polyhecamethylene biguanide
PMSF	Phenylmethylsulfonyl fluoride
RFLP	Restriction Frame length polymorphism
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
SSU	Short subunit
TAE	TRIS, acetic acid and EDTA buffer
TE	Tris-EDTA
VD	Vandamme broth

Chapter 1 Introduction

1.1 Amoebae

Amoebozoid organisms or amoebae are members of the kingdom Protista discovered in 1757 by Rösel Von Rosenhof. They can be found all around the world in soil or water environments. Amoebae play an essential role in the ecosystem releasing nutrients from bacteria, recycling biomass and making it available for plant absorption (Clarholm, 2002). They are not a monophyletic group as the term amoebae has no taxonomic value. Some amoebae are more closely related to animals and fungi than they are to other amoebae groups. According to Baldauf, eukaryotes can be divided into 4 groups: Unikonts, Archaeplastida, RAS (Rhizaria, Alveolates and Stramenopiles), and Excavates. Of these, all except Archaeplastida present amoebae (Baldauf, 2008). Figure 1 shows a simplified phylogenetic tree of the history of eukaryotes stressing the amoebozoid organisms and their diverse evolutionary origins.

While having a heterogeneous phylogenetic origin, they do share morphological and behavioural characteristics. All amoebae are characterized by their locomotion and morphology. Amoeboid locomotion can be described as a crawling-like movement that happens when extending cytoplasmic extensions, called pseudopodia (Khan, 2006). Pseudopodia can vary in shape and morphology in general, but its primary purpose is locomotion, and they can play a role in nutrition (Harvey et al., 2013).

The main groups containing amoebae are the Amoebozoa, Cercozoa and Heterolobosea. The Amoebozoa are characterized by the non-eruptive lobose or tube-like pseudopodia. They usually are uninucleated (Adl et al., 2005). Most taxa in Amoebozoa are free-living and include several amoebae, some amoeboflagellates, and social amoebae (Smirnov et al., 2005). Molecular data has shown that Ameobozoa also includes slime moulds (Bapteste et al., 2002).

Rhizaria includes groups that do not contain amoebae. However, they do present a large number of amoebae including chlorarachniophytes and *Paulinella chromatophora*, which are amoebae that are photosynthetic organisms (McFadden et

al., 1994; Nowack et al., 2008). Rhizarian amoebae are mainly testate (Cavalier-Smith & Chao, 2003).

Most heteroloboseans are amoebae with a flagellate state. They are naked amoebae abundant in the environment (Baldauf, 2008). Even though they are lobose like Amoebozoa, their pseudopodia move in a particularly “eruptive” manner (Pánek et al., 2017). *Naegleria* is the most important heterolobosean genus since it contains the human pathogen *Naegleria fowleri* (Carter, 1968, 1970). However, heteroloboseans also include vahlkampfiids, the terrestrial slime moulds Acrasids, among others (Pánek & Čepicka, 2012).

Amoebae exist in most eukaryote groups, even when they are most common in some clades. Nucleariids are a group of filose amoebae within the Opisthokonta at the boundary between fungi and animals. They include only three genera *Nucleari*a, *Fonticula*, and *Parvularia* (López-Escardó et al., 2017). Labyrinthulids are stramenopiles which form actin-rich large networks of pseudopods and which crawl. They prey on plants both in seawater where they feed on *Zostera* (a marine grass) and on land (Chitrampalam et al., 2015). *Chyrsophyceae*, the “golden algae” contains a number of amoeboid species which do not have cell walls in the amoeboid, motile phase. These include the genus *Synchroma* and *Chrysamoeba* (Hibberd, 1971; Patil et al., 2009).

Some amoebozoid organisms are predecessors of animals and fungi. *Capsaspora owczarzaki* is an amoeba that is the unicellular ancestor of metazoans, and is the link capable of explaining the unicellular prehistory of animals (Ferrer-Bonet & Ruiz-Trillo, 2017; Suga et al., 2013). Additionally, Rozellomycota is the most basal fungal lineage and includes the amoebal genus *Rozella* (Corsaro et al., 2014; Letcher et al., 2017). However, *Rozella* is not alone; chytrid fungi are also capable of amoeboid motility (Titus & Goodson, 2017).

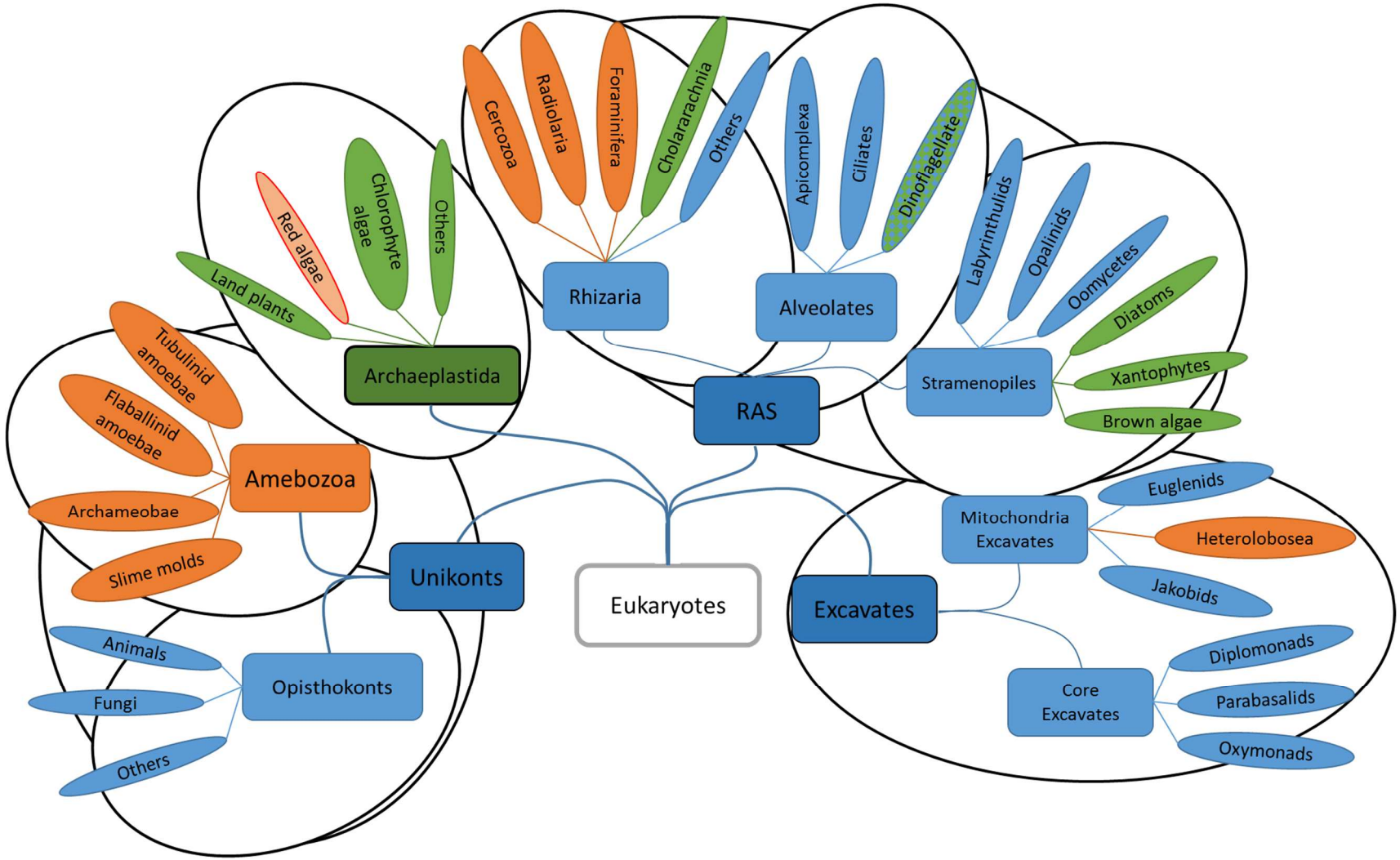


Figure 1 Summarized consensus phylogeny of Eukaryotes modified from Baldauf, 2008. Marked with orange are the groups of amoebozoid organisms. With green, red and brown are the photosynthetic organisms. In blue all other protists.

1.2 Free-living amoebae

Being such a diverse group, amoebae can be found in a great diversity of environments. The amoebae capable of surviving on their own in different environments without requiring a host are known as free-living amoeba (FLA). FLA have been known for over 200 years (Lorch, 1973). FLA have ubiquitous distribution, found in every continent, and in a very diverse group of environments.

However, despite the fact that these amoebae are free-living, some of them are facultative pathogens. Culbertson identified FLA as potential causatives of disease when he discovered a strain of *Acanthamoeba* that was capable of producing encephalitis in mice and monkeys (Culbertson et al., 1958). The most relevant FLA due to their potential to become pathogenic are *Balamuthia mandrillaris*, *Naegleria fowlerii* and species belonging to the genus *Acanthamoeba*. These amoebae are capable of producing an infection of the central nervous system known as amoebic encephalitis, where more than 95% of the cases are fatal (Harvey et al., 2013). Additionally, *Acanthamoeba* is capable of producing an eye infection called *Acanthamoeba* keratitis.

1.3 *Acanthamoeba*

Acanthamoeba are amoebozoid organisms of worldwide distribution and highly abundant in the environment (Geisen et al., 2014). They are characterized for their spine-like pseudopodia named acanthopodia (also referred to as acanthapodia), and their double-walled cysts.

1.3.1 Life cycle of *Acanthamoeba*

Acanthamoeba has asexual reproduction through binary fission. However, it has been suggested that they escape the damaging effect of Muller's (accumulating mutational load) ratchet though polyploidy (Maciver, 2016).

Acanthamoeba has a biphasic life cycle consisting of a vegetative trophozoite stage and a dormant and resistant cyst. When conditions are unfavourable, the trophozoite

goes through encystment, in which it encloses in a protective cyst that shows minimal metabolic activity (Siddiqui & Khan, 2012b). During this period, the external conditions are monitored by structures called ostioles. Each cyst has typically 3 or 4 ostioles (Weisman & Shaw, 1976). When conditions are favourable, trophozoites emerge from cysts. This process is called excystment and completes the life cycle of *Acanthamoeba*.

However, as new information becomes available, it appears that the simple biphasic life cycle is more complex. Another proposal suggests that *Acanthamoeba* has four different stages: trophozoite, precyst, immature cyst and mature cyst (Chávez-Munguía et al., 2013). These different stages help specify the intermediate degrees of encystment, in which the cell has different characteristics than the original two stages. Also, *Prostelium pyriformis* was shown actually to belong to the genus *Acanthamoeba* and renamed *A. pyriformis*. *A. pyriformis* is capable of forming a fruiting body or sporocarp. The sporocarp is formed by a non-cellular stalk, with a spore at the top and cannot form in aquatic environments. It is the first *Acanthamoeba* organism with this particular life cycle (Tice et al., 2016).

Acanthamoeba is capable of quickly developing a single-walled pseudocyst in the presence of solvent stress. Some of the solvents that have been identified and are capable of inducing pseudocyst formation are methanol, DMSO, acetone and propylene glycol (Kliescikova et al., 2011b, 2011a). This is particularly important because pseudocysts give *Acanthamoeba* a defence strategy against contact lens solutions (Kliescikova et al., 2011a). The pseudocyst differs from the cyst in genes that are expressed. For example, the cyst specific protein CSP21 is not expressed during pseudocyst differentiation (Hirukawa et al., 1998; Kliescikova et al., 2011b). Also, the expression of cellulose synthase I and II differs in timing and level of expression for cyst or pseudocyst (Kliescikova et al., 2011b).

The original reason identified for encystment was lack of food (Neff et al., 1964a). However, starvation is not the only identified reason for encystment. Some other causes include osmolarity (Cordingley et al., 1996), a variety of chemicals (Kilvington et al., 2008), temperatures, pH (Brindley et al., 2009) and even infection by some bacteria species such as *Francisella tularensis* (El-Etr et al., 2009). The double-walled cyst provides *Acanthamoeba* with protection to most conditions that the organism

might encounter in the environment including most pharmaceutical factors. *Acanthamoeba* cysts can survive more than 25 years frozen (Sriram et al., 2008).

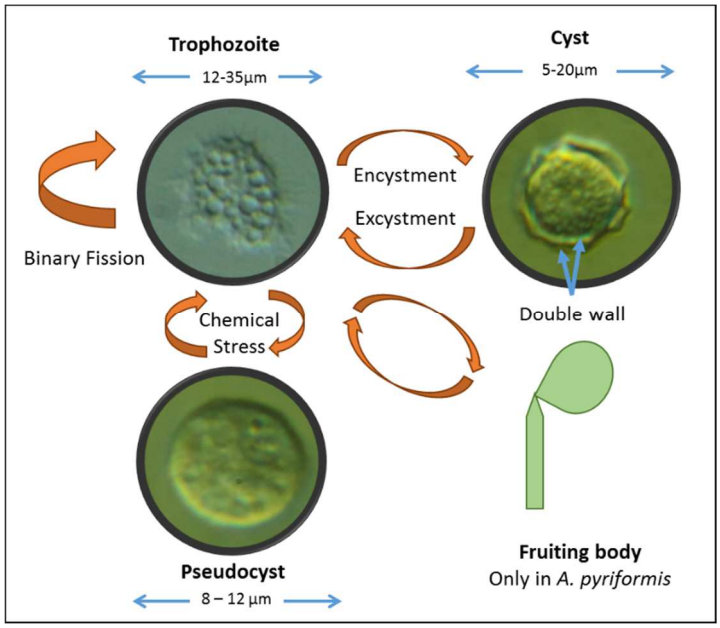


Figure 2. Simplified life cycle of *Acanthamoeba.* The image shows the encystment process and the ability to form single-walled pseudocyst during chemical stress. It also shows the ability of *Acanthamoeba pyriformis* to form a fruiting body or sporocarp.

Acanthamoeba has the capacity to encyst synchronously. Synchronous encystment is considered when more than 70% of the organisms encyst in 24 hours (Neff et al., 1964b). However, *Acanthamoeba* can lose the capacity to encyst synchronously when grown axenically. When this happens, the process takes longer and happens at different rates (Köhsler et al., 2008)

A simple diagram explaining the life cycle of *Acanthamoeba* is shown in Figure 2.

1.3.2 Morphology

Having two different life stages, their morphology is markedly different. Trophozoites are capable of changing their shape and are generally between 12 and 40 µm in diameter, but their size can change dramatically, depending on the species, genotype, the environment or their development. Cysts sizes range between 5 and 20 µm (Brindley et al., 2009; Lorenzo-Morales et al., 2013).

Acanthamoeba are uninucleate and polyploid. The nuclear chromosomes are small, can be numerous, and their sizes vary between 200 kb and 2 Mb (Byers et al., 1990; Rimm et al., 1988). *Acanthamoeba* contains between 1 and 2 pg/amoeba of DNA (Byers et al., 1990). The estimated size of the haploid genome is around 45 Mb (Byers, 1986). However, since *Acanthamoeba* is normally polyploid, the size of the total genome varies between organisms (Byers, 1986). The mitochondrial DNA presents a circular shape and a lower buoyant density than genomic DNA (Adam et al., 1969; Bohnert & Herrmann, 1974; Hettiarachchy & Jones, 1974). The genome of *Acanthamoeba castellanii* was published in 2013 (Clarke et al., 2013).

The plasma membrane has the particular characteristic of having lipophosphonoglycan which accounts approximately for 31% of the membrane's mass while being present on both sides of the membrane (Bowers & Korn, 1974; Korn, 1974). Actin, which consists of close to 20% of the total protein, myosin II and other cytoskeletal proteins are responsible for locomotion, cell transport and cell division (Khan, 2006).

Acanthamoeba has different types of vacuoles in the cytoplasm, a large contractive vacuole and smaller digestive vacuoles (Lorenzo-Morales et al., 2013). The protozoan contractile vacuole expels water for osmotic regulation and is capable of eliminating a volume of water equivalent to its total volume in 30 minutes (Bowers & Korn, 1968; Kitching, 1938). Myosin-I is known to be essential for contractile vacuole function and can be inhibited by monoclonal antibodies, causing the amoebae to swell and lyse (Doberstein et al., 1993).

The cyst consists of a double-layered wall, the endocyst and the ectocyst that are composed mainly of cellulose, which is lacking in the trophozoite and can account for 10% of the weight of the cyst (Blanton & Villemez, 1978; Tomlinson & Jones, 1962). Although the composition of the cysts varies from one specie to the other, it was first reported to be composed of 33% protein, 4-6% lipid, 35% carbohydrates, 8% ash and 20% unidentified materials (Neff et al., 1964a). While carbohydrates (cellulose) are present in both walls, the ectocyst has a high quantity of proteins (Chávez-Munguía et al., 2013; Hirukawa et al., 1998). During differentiation the carbohydrate composition of the amoeba changes. Trophozoite carbohydrates are composed

mainly of glucose (98%), while cysts have similar amounts of glucose and galactose (close to 45% each) among other carbohydrates (Dudley et al., 2009).

Although morphology used to be the primary criteria for classification, it was later almost totally discarded (Pussard & Pons, 1977). It was demonstrated that morphology was only useful for genus classification but had no phylogenetic relevance at a species level (Gast et al., 1996; Stothard et al., 1998; Yu et al., 2001).

1.3.3 Taxonomy

Taxonomic classification of amoebae is challenging since they possess ever-changing shape and a polyphyletic origin. *Acanthamoeba* is a genus of the Acanthamoebidae family from the Amoebozoa phylum. However, the higher taxonomy of the family changes slightly from author to author. Table 1 shows the different classifications of *Acanthamoeba* according to several authors and the National Center for Biotechnology Information (NCBI, 2015).

In 1930, Castellanii isolated an amoeba from a fungi culture of *Cryptococcus pararoseus*, now known as *Rhodotorula mucilaginosa*. The amoeba was identified as a new species and named *Hartmanella castellanii* by Douglas (Castellani, 1930; Douglas, 1930). One year later, Volkonsky, subdivided the genus *Hartmanella* into the three different genera (*Hartmanella*, *Glaeseria* and *Acanthamoeba*) and reclassified *H. castellanii* as *Acanthamoeba castellanii* (Volkonsky, 1931).

At first, the organisms in this genus were classified based on morphology. Initially, the mitotic spindle and the structure of the cyst were the primary determinants for classification (Volkonsky, 1931). However, classification based on morphological factors proved to be of little phylogenetic value. Therefore a new classification was required (Singh, 1950). In 1967, acanthopodia were described. The use of mitotic spindle for classification was discarded, and the structure of the cyst was given more importance in classification based on the doubled-wall found in *Acanthamoeba* which produces an endocyst and an ectocyst (Page, 1967). Later, the trophozoite shape and size was considered along with cyst structure for classification of the organisms. This new classification divided *Acanthamoeba* into three morphogroups (I, II and III) based on their morphological characteristics (Pussard & Pons, 1977). This method

of classification still has certain value as a quick method of classification of the amoebae. Group I presents stellate endocyst with clear delimitation between endocyst and ectocyst. Group II has typically smaller cysts that present different forms of endocyst but a wrinkled ectocyst with ostioles at the angle of the rays. Group III has rounded endocyst and a smooth, thin and delicate ectocyst (Lloyd, 2014; Page, 1967; Pussard & Pons, 1977). Group II is the most commonly found in the environment (Jeong et al., 2007).

	Corsaro, 2015	**Visvesvara, 1991**	**NCBI, 2015**	**Khan, 2006**
Superkingdom	Eukaryota			
Kingdom	Protozoa	Protista		Protista
Subkingdom	Sarcomastigota	Protozoa		
Phylum	Amoebozoa	Sarcomastigophora	Amoebozoa	Sarcomastigophora
SubPhylum	Lobosa	Sarcodina		Sarcodina
Superclass		Rhizopodea		Rhizpoda
Class	Discosea	Lobosea	Discosea	Lobosea
Subclass		Gymnamoebia		Gymnamoebia
Order	Centramoebida		Longamoebia	Amoebida
Suborder		Acanthapodina	Centramoebida	
Family	Acanthamoebidae			
Genus	*Acanthamoeba*			

Table 1. Comparison of taxonomic classification of *Acanthamoeba* (Corsaro et al., 2015; Khan, 2006; NCBI, 2015; Visvesvara, 1991).

Since 1996 the preferred basis for *Acanthamoeba* systematics has been the sequence of 18S ribosomal DNA (rDNA) short-subunit (SSU) (Gast et al., 1996). Presently there are 20 genotypes of *Acanthamoeba* based on 18S rDNA denominated T1 to T20. Nevertheless, the classification is continuously in flux (Corsaro et al., 2015). There are several species names for a single genotype and some species names that are used for different genotypes. One example of the challenges found in *Acanthamoeba* classification is genotype T99. Several environmental sequences were included in T99, but no organisms existed in culture (Fuerst et al., 2015). However, the 18S rDNA sequence from genotype T99 proved to be an artefact, with a middle fragment belonging to an *Acanthamoeba* T13, but the 5' and 3' end-fragments belonged to a nematode and a cercozoan respectively (Corsaro & Venditti,

2017).The different species and their relation to the 20 genotypes are shown in Table 2.

1.3.3.1 Isoenzyme profile

Before sequencing of the 18S became practical, several other methods were used to determine *Acanthamoeba* phylogeny. Researchers have proposed the use of isoenzymes as a form of classification. Costas and Griffiths proposed the use of esterases and acid-phosphatases for classification (Costas & Griffiths, 1980). Later on, they proposed some other enzymes that didn't show conclusive phylogenetic value (Costas & Griffiths, 1985). Meanwhile, De Jonckheere also used isoenzymes as a form of classification. He compared acid-phosphatase, leucine amino peptidase, malate dehydrogenase, propionyl-esterase, glucose phosphatase isomerase, phosphoglucomutase and alcohol dehydrogenase in addition to protein patterns to clarify some aspects of *Acanthamoeba* morphology (Jonckheere, 1983). In 1995, Kong proposed the comparison of isoenzyme profiles and mitochondrial DNA (mtDNA) fingerprints. He proposed the extraction of mtDNA and digestion as a method for strain identification (Kong et al., 1995).

1.3.3.2 Riboprinting

Kong suggested the use of restriction fragment length polymorphism (RFLP) of amplified SSU fragments (Kong & Chung, 1996). Later, Chung et al. proposed a PCR based restriction analysis of the SSU which appeared to be closely related to the morphogroups proposed by Pussard and Pons (Chung et al., 1998; Pussard & Pons, 1977). In 2001, Yu used both PCR-RFLP of the SSU as well as mtDNA fingerprint to identify the strains of isolates obtained from contact lens storage cases from Korea. Most of the samples showed similar profiles as other known pathogenic strains (Yu et al., 2001).

Genotype	Species related			AK	GAE	MG	References
T1	*A. castellanii*			No	Yes	II	(Gast et al., 1996; Stothard et al., 1998)
T2	*A. palestiniensis*	*A. polyphaga*	*A. pustulosa*	Yes	Yes	III	(Gast et al., 1996; Stothard et al., 1998)
T3	*A. griffini*	*A. pearcei*		Yes	No	II	(Gast et al., 1996; Stothard et al., 1998)
T4	*A. castellanii*	*A. polyphaga*	*A. royreba*	Yes	Yes	II	(Gast et al., 1996; Stothard et al., 1998)
	A. ludgunensis	*A. triangularis*					
T5	*A lenticulata*			Yes	Yes	III	(Gast et al., 1996; Stothard et al., 1998)
T6	*A. hatchetii*	*A. palestiniensis*		Yes	No	III	(Gast et al., 1996; Stothard et al., 1998)
T7	*A. astronyxis*			No	No	I	(Gast et al., 1996; Stothard et al., 1998)
T8	*A. tubiashi*			No	No	I	(Gast et al., 1996; Stothard et al., 1998)
T9	*A. comandoni*			No	No	I	(Gast et al., 1996; Stothard et al., 1998)
T10	*A. culbertsoni*			No	Yes	III	(Gast et al., 1996; Stothard et al., 1998)
T11	*A. stevensoni*	*A. hatchetii*		Yes	No	II	(Gast et al., 1996; Stothard et al., 1998)
T12	*A. healyi*			No	Yes	III	(Gast et al., 1996; Stothard et al., 1998)
T13				Yes	No	II	(Hewett et al., 2003; Horn et al., 1999)
T14				No	No	III	(Gast et al., 1996; Hewett et al., 2003)
T15	*A. jacobsi*			Yes	No	III	(Hewett et al., 2003)
T16				No	No	II	(Corsaro & Venditti, 2011)
T17				No	No	I	(Nuprasert et al., 2010)
T18	*A. byersi*			No	Yes	I	(Qvarnstrom et al., 2013)
T19	*A. micheli*			No	No	II	(Corsaro et al., 2015)
T20				No	Yes	II	(Fuerst et al., 2015; Visvesvara et al., 2007)

Table 2. Genotypes and species of *Acanthamoeba*. The 20 genotypes are presented with the different species names found in the literature. AK and GAE columns show if the strain has been reported to cause the disease. The MG column refers to the morphogroup of each strain

1.3.3.3 **18s rDNA**

In 1987, Woese proposed the ribosomal DNA as ultimate molecular clock measuring evolutionary divergence (Woese, 1987). That same year, Sogin and Gunderson (1987) tried to establish the phylogeny of the eukaryotes based on the 18S and 20S.

The 18S gene used for the identification of eukaryotic species is over 2kb and varies depending on the species and the genotype. In the case of *Acanthamoeba*, the three morphological subgroups can still be divided by the sequence of the 18S rDNA. According to Stothard et al., subgroups II and III represented four different genotypes T1-T4, while subgroup I represented all the other genotypes known until then (T5-T12) (Stothard et al., 1998). However, the classification of each strain depending on the morphogroups varies depending on the author.

Subgroups II and III usually have 18S sequences close to 2300 bp while subgroup I has a more extended sequence close to 2700 bp (Stothard et al., 1998). Nevertheless, as more information has become available, this has changed. In 1996, *A. griffini* was identified from an AK patient, and the 18S was measured at around 2800 bp with a unique morphogroup I intron despite being characterized as a member of morphogroup II (Ledee et al., 1996). Later, similar problems were encountered with some isolates of the T5 *A. lenticulata. A. lenticulata* presented 18S gene fragments of around 300 bp and introns characteristic of subgroup I despite being a member of morphogroup III (Schroeder-Diedrich et al., 1998). However, as seen in Table 2, the most current classification of morphogroups has changed (Corsaro et al., 2015).

Due to the disparity of sequences from the different subgroups, a smaller diagnostic fragment (ASA.S1) was proposed as a highly reliable tool for quick identification. The ASA.S1 fragment is around 450 bp (Schroeder et al., 2001). Later, Booton proposed an even smaller fragment from the ASA.S1 region of around 113 bp as the critical part for identification of *Acanthamoeba* strains (Booton et al., 2002).

Despite all the different fragments proposed for classification, the full 18S gene is necessary for correct identification. Smaller fragments can be indicative of a new lineage, but they are not conclusive as they lack information only available in the full gene for the establishment of new genotypes (Corsaro et al., 2015). At the moment,

at least 5% difference in the 18S rDNA is required to define a new genotype (Fuerst et al., 2015).

It has been determined that *Acanthamoeba* phylogeny is closer to plants and animals than some other amoebozoid organisms. The study of the 18S sequence has shown a more recent phylogenetic branch and origin for *Acanthamoeba* than other amoebae such as *Naegleria*, which originated from a very different branch of eukaryotic evolution (Clark, 1990; Clark & Cross, 1987).

In *Acanthamoeba*, rDNA is located in the nucleus and the mitochondria (Bradley & Marciano-Cabral, 1996). Other amoebae such as vahlkampfiids have the rDNA located exclusively on an extrachromosomal plasmid (Clark, 1990).

1.3.3.4 Cytochrome oxidase subunit I

Cytochrome oxidase subunit 1 (COI) barcoding is a recent approach to studying the phylogeny of amoebae. It is based on the sequencing and analysis of the COI gene, which has shown to give consistent results comparable to 18S rDNA in *Acanthamoeba* (Crary et al., 2011). The COI barcoding has been used with other species including testate amoebae (Kosakyan et al., 2012). In some groups such as the genus *Vannella*, sequencing the COI gene is essential for the proper identification of the species as the 18S can be inadequate (Nassonova et al., 2010). However, further studies and more sequences are required to confirm the value of COI as a tool of identification.

A simple timeline of *Acanthamoeba spp*. taxonomic history is presented in Figure 3.

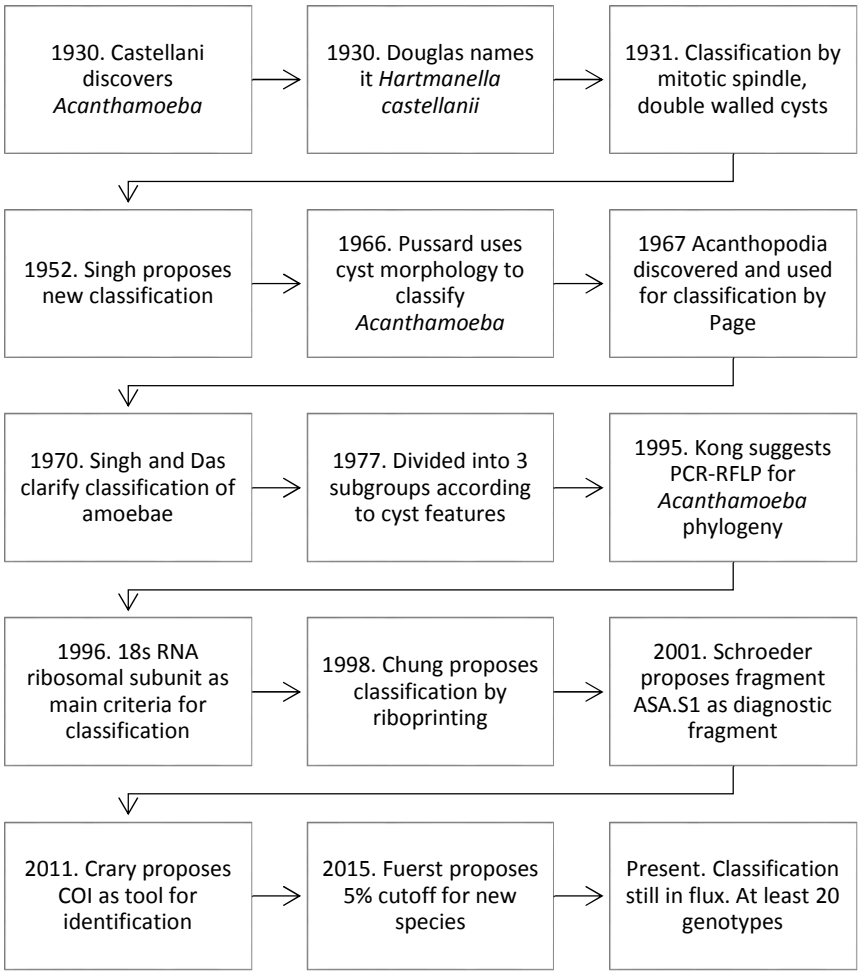


Figure 3. Brief taxonomic history of *Acanthamoeba* classification.

1.3.4 Locomotion

Acanthamoeba organisms inhabit different environments, mainly of soil or water. These environments usually are substrates or the water-air interface (WAI) of aquatic environments. Depending on the environment, their locomotion slightly varies. *Acanthamoeba* normally moves directionally. However, when inhabiting the WAI, the amoebae tend to use passive locomotion. It is important for *Acanthamoeba* to be able to inhabit the WAI, as it enables them to move through aerosols and increases the potential of infecting other organisms (Preston et al., 2001). *Acanthamoeba* moves relatively fast in relation with other protozoa at around 0.75 µm/sec either in solid substrate or the water-air interface (Preston et al., 2001).

Like in most protozoa, locomotion in *Acanthamoeba* occurs at a cellular and sub-cellular level (Preston & King, 1984). At a cellular level, *Acanthamoeba* presents a fluid-like structure forming a hyaline zone or pseudopodium at the front. These pseudopodia present acanthopodia and lack cytoplasmic granules (Bowers & Korn, 1968; Preston & King, 1984). The pseudopodium and acanthopodia contain hyaline cytoplasm, which is composed of only the most basic cytoplasmic components and microtubules (Bowers & Korn, 1968). Another two basic components of *Acanthamoeba* locomotion are myosin I and actin (Baines et al., 1992; Gordon et al., 1976). Actin filaments are mostly concentrated at the edge of the amoebae, sitting close to the membrane, and are responsible for the cellular protrusion characteristic of the amoebic movement. *Acanthamoeba* locomotion has been extensively studied as model organisms with an emphasis on the actin cytoskeleton based-motility (Pollard et al., 1989).

1.3.5 Feeding systems

Acanthamoeba feeds on microorganisms through phagocytosis and pinocytosis (Bowers & Olszewski, 1972; Weisman & Korn, 1967). They feed mainly from bacteria, but can also feed on yeast and algae. Organisms ingested by *Acanthamoeba* are digested in phagolysosomes (Allen & Dawidowicz, 1990; Bowers & Olszewski, 1983; Khan & Siddiqui, 2014; Weekers & Drift, 1993). *Acanthamoeba* is capable of distinguishing digestible and indigestible particles once they have been internalized and is capable of expelling indigestible particles (Bowers & Olszewski, 1983). Acanthopodia play an essential role in capturing prey (Lorenzo-Morales et al., 2013).

Phagocytosis is a process dependant on receptors. A mannose binding protein mediates the binding of *Acanthamoeba* to food organisms and subsequent phagocytosis (Allen & Dawidowicz, 1990; Alsam et al., 2005a; Garate et al., 2004). During phagocytosis, amoebae engulf prey into a phagolysosome, where it is killed by acidification, oxidative stress, deprivation of nutrients and diverse antimicrobials (Haas, 2007; Strassmann & Shu, 2017).

Pinocytosis is non-specific and happens through invagination of the membrane. It is used when large solutes and food particles are the nutrients of choice (Bowers & Olszewski, 1972; Khan, 2006).

During infection of mammals, *Acanthamoeba* feeds through cup-shaped structures called amoebostomes in a process called trogocytosis. During trogocytosis, the cell cuts and ingests small pieces of the host cell (Lorenzo-Morales et al., 2013). *Acanthamoeba's* amoebostomes have varying sizes depending on the function, where large ones help degradation of the target cell and smaller ones tear small parts (González-Robles et al., 2009; Khan, 2001; Khan & Tareen, 2003).

1.3.6 Ecology

It is common to find *Acanthamoeba* in an extensive range of ecosystems such as in soil and water bodies (Behniafar et al., 2015; Reyes-Batlle et al., 2014), but can also be found in air conditioning system (Astorga et al., 2011), tap water (Bagheri et al., 2010; Jeong et al., 2007), water bottles (Maschio et al., 2014; Rivera et al., 1981), humidifiers (Tyndall et al., 1995) sewage (Sawyer et al., 1992), compost (Conza et al., 2013), sediments soil (Reyes-Batlle et al., 2014; Sawyer et al., 1992), animal faeces (Rezaeian et al., 2008), beaches (Lorenzo-Morales et al., 2005b), surgical instruments (Rezaeian et al., 2008), dialysis units (Hassan et al., 2012), eye wash units (Paszko-Kolva et al., 1991), dental units (Hassan et al., 2012), contact lenses and several human tissues (Khan, 2006). Therefore, most individuals have been exposed to the organism as shown by anti-*Acanthamoeba* antibodies found in the vast majority of individuals regardless of gender or nationality (Brindley et al., 2009; Cursons et al., 1980).

In the environment, amoebae play a vital role in the composition of the microbial communities and nutrient cycling. Amoebae are the primary bacterial predators in soil and are capable of accounting for 60% of the bacterial decrease (Clarholm, 1981; Elliott & Coleman, 1977). When feeding on bacteria, amoebae are capable of metabolizing nitrogen playing an essential role in nitrogen mineralization. Otherwise, nitrogen would be immobilized by bacteria and unavailable for plants. Therefore, amoebae are essential for soil fertility. (Weekers et al., 1993; Weekers & Drift, 1993; Woods et al., 1982). Additionally, this process helps with the regulation of bacterial populations.

1.4 Bacteria-Amoebae interactions

As with any interaction found in nature, the relationship between amoebae and bacteria have shaped both organisms as they continuously adapt to each other. Even though amoebae are responsible for most of the predation of bacteria in the environment, it is not the only interaction between these organisms. Bacteria-amoebae interactions can be classified into three different types: relations in which *Acanthamoeba* benefit at the expense of bacteria; relations in which bacteria benefit at the expense of amoebae; and mutualistic relations in which both benefit. There can be a fourth type relation in which they compete for the limited amount of resources, as it happens for all organisms sharing the same environment.

1.4.1 Amoebae prey on bacteria

Acanthamoeba feeds mainly from preying on bacteria. It is responsible for the elimination of 60% of the bacterial populations in the environment (Rosenberg et al., 2009). *Acanthamoeba* has shown a preference for gram-negative bacteria, despite the abundance of gram-positive bacteria found in the environment (Khan & Siddiqui, 2014; Weekers et al., 1993). When given a choice, *Acanthamoeba* prefers to feed on gram-negative bacteria such as *Escherichia coli* and *Kleibsella aerogenes*. However, it is capable of feeding and growing in several species of bacteria including *Agrobacterium tumefaciens, Bacillus megaterium, Bacilus subtilis, Chromatium vinosum, Micrococcus luteus, Pseudomonas fluorescens*, and *Serratia marcescens* (Weekers et al., 1993).

1.4.2 Bacteria invade amoebae

Some bacteria have developed adaptations to avoid digestion by *Acanthamoeba*, and survive inside of it. This produces a system in which *Acanthamoeba* harbours bacteria that would otherwise have trouble surviving in the environment providing them with a safe place to proliferate (Greub & Raoult, 2004; Guimaraes et al., 2016; Strassmann & Shu, 2017). Therefore, *Acanthamoeba* often has obligate intracellular symbionts (Schmitz-Esser et al., 2004). The primary mechanism to avoid phagocytosis is subverting the antimicrobial mechanisms of the phagosome. However, some bacteria

are also capable of escaping vacuoles and invading the cytoplasm, which provides ideal characteristics for bacterial growth (Strassmann & Shu, 2017).

Bacteria capable of surviving within amoebae can be divided between those capable of reproducing, and those that remain intracellular without multiplying (Siddiqui & Khan, 2012c). The bacteria capable of reproducing such as *Bacillus anthracis*, *Chlamydia pneuminiae*, *Parachlamydia acanthamoebae* and *Ralstonia pickettii* are considered pathogens of *Acanthamoeba* as they cause lysis killing the host (Amann et al., 1997; Dey et al., 2012; Essig et al., 1997; Michel & Hauröder, 1997). However, before lysis, *Acanthamoeba* serves as a reservoir for the bacteria, allowing it to reproduce, survive, invade other organisms such as humans, and cause disease (Greub & Raoult, 2004).

However, not all bacteria that can survive inside *Acanthamoeba* are capable of reproducing. These adaptations give the bacteria the capacity to survive harsh environments, use *Acanthamoeba* as a vector of transmission and help them develop strategies to evade vertebrate immune system that can be similar to the amoebae (Khan & Siddiqui, 2014; Siddiqui & Khan, 2012c) .

The relationship between *Acanthamoeba* and different bacteria might change depending on environmental factors. For example, *L. pneumophila* produces lysis of *Acanthamoeba* at 37˚C, but it is phagocytized and digested at temperatures around 20˚C (Anand et al., 1983).

Whether they multiply or not, some bacteria use *Acanthamoeba* as Trojan horses since amoebae serve as a vector for the bacteria to infect other organisms. Until 2007, only *Acanthamoeba* species of morphogroup II have been known to carry bacterial endosymbionts, probably due to their genetic and epidemiological capabilities (Jeong et al., 2007). Figure 4 shows an example of bacteria inside a cyst.

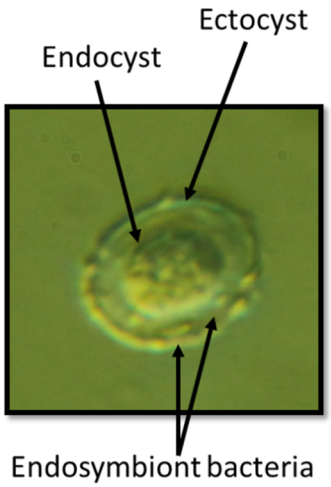


Figure 4. Bacteria inside *Acanthamoeba* cyst.

1.4.3 Mutualism

There are aspects of the relation where both the amoebae and the bacteria benefit. For bacteria, intra-amoebal growth can enhance resistance to antibiotics and biocides (Barker et al., 1995; Walochnik et al., 1999). Interaction with amoebae can also increase virulence and pathogenicity (Cirillo et al., 1997, 1999). Moreover, some bacteria are capable of taking some amoebic characteristics like the amoebic membrane proteins found in *L. pneumophilla* after growing inside amoebae (Barker et al., 1993). Additionally, inter-kingdom lateral gene transfer occurs commonly between amoebae, bacteria and viruses (Clarke et al., 2013; Moliner et al., 2009). *Acanthamoeba* growing in the presence of bacteria have a higher virulence than those growing axenically (Larkin et al., 1991).

Table 3 shows a list of bacteria capable of surviving within *Acanthamoeba.*

Bacteria	Localization in *Acanthamoeba*	More information	Human Disease	Reference
Bacillus anthracis	Phagosome	Produces lysis	Anthrax	(Dey et al., 2012)
Candidatus Nucleicultrix amoebiphila	Nucleus	Produces lysis in *A. castellanii*		(Schulz et al., 2014)
Chlamydia pneumoniae	Vacuoles	Can produce lysis	Pneumonia	(Essig et al., 1997; Michel & Hauröder, 1997)
Coxiella burnetti	Vacuoles	Forms spore-life structures	Q fever	(La Scola & Raoult, 2001)
Escherichia coli	Vacuole		Digestive tract infection	(Alsam et al., 2006; Barker et al., 1999)
Francisella tularensis	Vacuoles	Can survive for 3 weeks	Tularaemia	(El-Etr et al., 2009)
Helicobacter pylori	Vacuoles		Asymptomatic disease	(Moreno-Mesonero et al., 2016)
Legionella pneumophilla	Throughout cyst and trophozoite	Enters via phagocytosis	Legionnaire's disease	(Cirillo et al., 1994; Kilvington & Price, 1990)
Listeria monocytogenes	Phagosome and cytoplasm	Does not survives inside cysts	Listeriosis	(Chau & Muller, 1990; Zhou et al., 2007)
Mycobacterium avium	Within cyst walls		*M. avium*-intracellular infection	(Cirillo et al., 1997; Steinert et al., 1998)
Mycobacterium leprae	Acid rich regions (lysosomes)		Leprosy	(Wheat et al., 2014)

Paenibacillus spp.		Emergent pathogen		(Maschio et al., 2015)
Parachlamydia acanthamoebae	Vacuole	Lysis at higher temperature	Respiratory infections	(Amann et al., 1997; Greub & Scola, 2003)
Pseudomona aureoginosa		Inhibits growth of *Acanthamoeba*	Several	(Qureshi et al., 1993)
Ralstonia pickettii	Vacuoles	Produces amoebal lysis	Several infections	(Michel & Hauröder, 1997)
Sarcobium lyticum (Legionella lytica)	Vacuole and cytoplasm	Obligate parasite	Respiratory infections	(Drozanski, 1991; Springer et al., 1992)
Salmonella typhimurium	Contractile vacuole	Role in bacterial translocation	Salmonellosis	(Gaze et al., 2003)
Staphiloccocus aureus	Phagosomes and cytoplasm		Several	(Huws et al., 2006, 2008)
Taylorellae equigenitalis	Cytoplasm	Equine genital pathogen		(Allombert et al., 2014)
Vibrio cholerae	Cytoplasm		Cholera	(Abd et al., 2007)
*Vibrio parahaemolyticus**	Requires presence of amoebae but cannot survive inside		Gastroenteritis	(Laskowski-Arce & Orth, 2008)

Table 3. Bacteria capable of surviving intracellularly in *Acanthamoeba*. The table also shows the localization of the bacteria within *Acanthamoeba*, comments and which human disease they cause when applicable. *In the case of *V. parahaemolyticus*, it does not live intracellularly, but requires the presence of *Acanthamoeba.*

1.4.4 Bacterial infections aided by *Acanthamoeba*

Acanthamoeba provides several advantages for bacteria that have developed adaptations to survive inside it. *Acanthamoeba* offers an environmental niche that bacteria can use for survival. Moreover, the amoeba aids in transport helping in the transmission and infection of humans and animals (Khan & Siddiqui, 2014). Some of the diseases caused by bacteria carried through *Acanthamoeba* can be seen in Table 3.

1.4.5 *Legionella pneumophila*

L. pneumophila is ubiquitous in freshwater environments, where it replicates within protozoan hosts (Fields et al., 2002). *L. pneumophila* is the causative agent of Legionnaires' disease (LD). LD is an atypical form of pneumonia first described after an outbreak in Philadelphia in 1976 (Fraser et al., 1977). Modern technologies, such as air conditioning systems, can cause aerolization of water and parasites such as *Acanthamoeba* carrying *L. pneumophila*. Once inhaled, *L. pneumophila* infects alveolar macrophages (Horwitz, 1983). The development of the infection causes LD. In 2012, Edinburgh had an LD outbreak with 61 positive cases causing the death of four people. It was the largest outbreak in the UK in over a decade costing the health system an estimated £725,800 (Irons et al., 2013).

L. pneumophila has long been associated with *Acanthamoeba*. Rowbotham suggested that the source of disease are vesicles and amoebae carrying the bacteria as vectors, instead of free-living bacteria (Rowbotham, 1980). *L. pneumophila* organisms that grow inside *Acanthamoeba* have shown increased virulence and ability to invade the host (Cirillo et al., 1994, 1999). Furthermore, *L. pneumophila* grown in the presence of *Acanthamoeba* has shown increased resistance to antimicrobials (Barker et al., 1995).

1.5 Interactions between amoebae and other organisms

1.5.1 Virus

It is important to consider that also viruses, including giant viruses, are capable of infecting *Acanthamoeba* (Phillipe et al., 2013). Some of these viruses are also capable of gene transfer within *Acanthamoeba,* with either the amoeba or intra-cellular bacteria (Moliner et al., 2009). The main viruses infecting *Acanthamoeba* are shown in Table 4.

Virus	Comment	Reference
Adenoviruses	Enteritic, respiratory and eye infections	(Lorenzo-Morales et al., 2007)
Pandoraviruses	Giant virus	(Phillipe et al., 2013; Scheid et al., 2014)
Mimivirus	Giant virus	(La Scola et al., 2003; Zauberman et al., 2008)
Coxsackievirus	Causes aseptic meningitis	(Mattana et al., 2006)
Mamavirus	Is a different Mimivirus strain	(Colson et al., 2011)
Mollivirus sibericum	Giant virus found infecting in permafrost	(Legendre et al., 2015)
Pithovirus	Giant virus found infecting in permafrost	(Abergel & Claverie, 2014)
Marseillevirus	Evolutionary evidence through gene analysis	(Boyer et al., 2009)

Table 4. List of virus capable of surviving inside *Acanthamoeba*.

1.5.2 Fungi

At least one fungal organism has been reported to be able to survive inside *Acanthamoeba*. *Cryptococcus neoformans* can replicate inside *Acanthamoeba* without being degraded (Neilson et al., 1978). The interaction of *C. neoformans* and *Acanthamoeba* might explain the virulence of the fungi and its capacity to invade mammalian cells (Steenbergen et al., 2001),

1.6 Diseases and importance

One of the main reasons for *Acanthamoeba* importance is its relationship with human health and its ability to cause disease.

While some strains are innocuous, others are facultative pathogens for humans and animals (Martinez, 1980). Several species such as *A. lenticulata* and *A. polyphaga* present pathogenic and non-pathogenic organisms (Khan & Tareen, 2003). The easiest way of identifying potential pathogenicity of a strain is testing its tolerance to higher temperatures (37˚C) and osmolarity (De Jonckheere, 1980; Khan & Tareen, 2003).

More than 80% of individuals present anti-*Acanthamoeba* antibodies, meaning most people have been in contact with the amoeba (Chappell et al., 2001). However, it is not clear if the antibodies are protective (Visvesvara et al., 2011). Infections by *Acanthamoeba* mostly occur to contact lens wearers or immune deficient patients.

Mainly, *Acanthamoeba* causes two well-recognized infections: Granulomatous amoebic encephalitis (GAE) and *Acanthamoeba* keratitis (AK). Most people do not develop any disease from the exposure to *Acanthamoeba*. However, immune compromised patients are at a high risk of developing an infection (Chappell et al., 2001). The immune deficient patients infected with *Acanthamoeba* usually have AIDS or recently had a transplant. The first reported infection in a patient with AIDS was in 1986 (Gonzalez et al., 1986). Additionally, immunocompromised patients can develop other infections such as *Acanthamoeba* rhinosinusitis and cutaneous acanthamebiasis.

1.6.1 Granulomatous amoebic encephalitis (GAE)

GAE is a disease caused when amoebae infect the central nervous system (CNS). GAE has a high mortality rate. The first reported case occurred in the United States in 1972 to a patient with Hodgkin lymphoma (Jager & Stamm, 1972). *Acanthamoeba* is the leading causative agent of GAE. However, it is not the only one. In 1990, *Balamuthia mandrillaris* was discovered when it was isolated from the brain of a mandrill-baboon. *B. mandillaris* is capable of causing GAE (Visvesvara et al., 1990).

As with most *Acanthamoeba* infections, GAE happens typically in immunocompromised patients, but it is not exclusive (Visvesvara et al., 2007). Usually, the amoebae enter the host through the pulmonary tract or skin lesions. Then, the infection travels by haematogenous dissemination, until it reaches the CNS invading the blood-brain barrier (Martinez, 1991). Even though it has an unknown incubation period, the development is slow, occurs through several weeks, and can span several months. However, once the infection reaches the CNS, it usually results in death within days or weeks (Khan, 2006; Martinez, 1991). Since it starts with infection of the pulmonary tract of skin lesions, GAE is closely related to other *Acanthamoeba* infections such as *Acanthamoeba* rhinosinusitis and cutaneous acanthamebiasis.

GAE causes a pro-inflammatory response, and neuronal damage (Khan, 2006). The symptoms commonly include headaches, stiff neck, nausea, vomiting, fever, lethargy, visual disturbances, hemiparesis, seizures, mental-state changes, facial nerve palsy, ataxia and other cerebellar signs (Crary et al., 2011; Visvesvara et al., 2007).

Diagnosis is difficult, as imaging techniques, such as MRI, do not give conclusive results. Results from imaging techniques are similar to several other diseases like brain abscesses, tumours and cerebrovascular accidents (Visvesvara et al., 2011). The only way to verify is through cultivation and PCR. Unfortunately, cultivation usually happens from post-mortem biopsies (Visvesvara et al., 2011).

Since diagnosis is complicated and most cases are identified post-mortem, treatment options are limited. However, some cases, where early diagnosis was possible, were successfully treated through different combinations of drugs that included pentamidine, voriconazole, isethionate, sulfadiazine, fluconazole chlorexidine gluconate, ketoconazole and miltefosine (Aichelburg et al., 2008; Martinez et al., 2000; Slater et al., 1994; Webster et al., 2012). GAE presents the challenge of drug delivery as most of the drugs used in infections, are incapable of crossing the blood-brain barrier (Visvesvara, 2010).

1.6.2 *Acanthamoeba* rhinosinusitis

Rhinosinusitis is a disorder characterised by the inflammation of the nasal passages. As it is the case with other *Acanthamoeba* infections, it occurs in immunocompromised patients and usually is fatal (Rivera & Padhya, 2002). Most cases are related to HIV infection, but at least two cases have been reported in immunocompromised patients from lung transplants (Cammaroto et al., 2015).

Until 2015, nineteen cases had been reported of *Acanthamoeba* rhinosinusitis (Cammaroto et al., 2015). The diagnosis of *Acanthamoeba* rhinosinusitis is difficult as it is extremely rare and mimics the development of similar diseases (Dickson et al., 2009). There is no specific treatment.

1.6.3 Cutaneous acanthamebiasis or granulomatous dermatitis

Acanthamoeba is capable of producing skin infections, and it usually appears as a complication in immunocompromised patients, especially, but not limited, to those with AIDS. Nodules and skin lesions are characteristic of the infection (Khan, 2006).

Acanthamoebiasis can co-occur with GAE or with sinus involvement (Gonzalez et al., 1986; Helton et al., 1993; Slater et al., 1994). Early diagnosis and treatment are vital due to the high risk of dissemination and development of GAE (Walia et al., 2007).

1.6.4 *Acanthamoeba* keratitis (AK)

AK is a sight-threatening infection of the cornea that mostly affects individuals that wear contact lenses or had corneal trauma. AK is a relatively new disease that first appeared in a Texan rancher in 1973, and was first reported in 1974 in the United Kingdom (Jones et al., 1975; Nagington et al., 1974). In 1985, the relation between contact lenses and the disease was firmly established (Moore et al., 1985). Ever since, AK importance has been growing with the increased use of contact lenses. However, AK is still considered a rare disease included in the ORPHANET database (ORPHA:67043), with an estimated prevalence of 1-9/100000 (Lorenzo-Morales et al., 2015).

AK infection is very painful and can be persistent. There is no specific treatment. Even with medical intervention, there is no guarantee of success, and the eye may be lost. New drugs/treatments are urgently needed (Lorenzo-Morales et al., 2015). Besides pain, patients experience photophobia, redness caused by hyperaemia, eyelid ptosis, ulcers and infiltrate causing cloudiness in the eye in later stages (Clarke & Niederkorn, 2006). In more advanced cases, keratoplasty might be the only option of treatment to improve the chances of recovery (Hammersmith, 2006). Ordinarily, only one eye is infected, but there have been some reported cases of bilateral keratitis (Cachia Markham & Mercieca, 2012; Rama et al., 2003; Visvesvara et al., 2007).

Unlike other *Acanthamoeba* infections that generally happen due to immunosuppression, contact lens use is the primary risk factor for AK. AK is most often a result of deficient hygiene in the storage and handling of the contact lenses. The frequent presence of bacterial biofilms in contact lenses facilitates the growth and infection of *Acanthamoeba* (Simmons et al., 1998). However, there are other factors involved in AK infection such as corneal trauma. This means that AK can occur in non-contact lens wearers.

1.6.4.1 Contact lens wear

Mostly, AK infection is related to soft contact lens wear. The first documented attempt to develop contact lenses was made by August Muller in 1889 trying to correct his myopia, where he described the principle used today. He correctly hypothesised that the forces of adhesion and capillarity would hold the lens in place (Key, 2007). Rigid contact lenses started between 1938- 1940. Soft contact lenses appeared in 1971. Since then, the number of contact lens wearers has increased. In the UK, the number of contact lens wearers was estimated around 4.2 million, representing close to 9 percent of the adult population aged 15-64 years old (Optometry Today, 2016). By 2015, an estimated 40.9 million adults in the United States were contact lens wearers, out of which 93% reported wearing soft contact lenses (Cope et al., 2015). According to the American Optometric Association, 80% of the wearers use daily soft contact lenses and 15% use extended wear. Around 50% of the wearers wear 1 to 2 week contact lenses (American Optometric Association, 2003).

Between 40% and 90% of contact lens wearers do not follow proper cleaning procedures (Bui et al., 2010). Cope reported that close to 99% of contact lens wearers have at least one behaviour that puts them at risk of infection (Cope et al., 2015).

1.6.4.2 Keratitis distribution and growth

Since its discovery in 1974, the apparent incidence of AK has constantly been growing due to the increased use of contact lenses as well as the increased awareness and improvement of diagnostic techniques that lead to a higher report rate. Since the 1980s, there has been a dramatic rise in AK cases parallel to the increase in popularity of soft contact lenses (Stehr-Green et al., 1989).

AK is potentially endemic to all countries. It can be assumed that the reason for countries without any reported cases of AK, is due to limitations in research rather than to the absence of *Acanthamoeba*. However, it is a disease more prevalent in developed countries due to the higher use of contact lenses. Nevertheless, AK is not exclusive to developed countries as evidenced by cases of AK all throughout the world. The first cases in America and Europe were in the mid-70s. In 1984, the first reported case of AK in Oceania was reported in a 19-year-old patient from Australia (Jackson et al., 1986). The first case reported in Africa was in 1990 in Mali (Resnikoff et al., 1991). More cases are reported every year. Figure 5 shows a comparison of reported cases of AK between 1987 and 2017 (Auran et al., 1987). It is assumed that AK and *Acanthamoeba* are present worldwide. However, the countries shown are the ones where evidence in the literature was found for AK.

In the UK, 20 cases per million contact lens wearers occur. The incidence is thought to be higher than in other countries of the world (15 times the estimate for the United States and 7 times for Holland) due to the storage of domestic tap water (Kilvington et al., 2004; Radford et al., 2002). Country wise the incidence varies between 1-33 cases per million, but it has been reported as high as 149 cases for the west of Scotland, increase which is normally attributed to the water systems (Malla & Goyal, 2016; Seal et al., 1999).

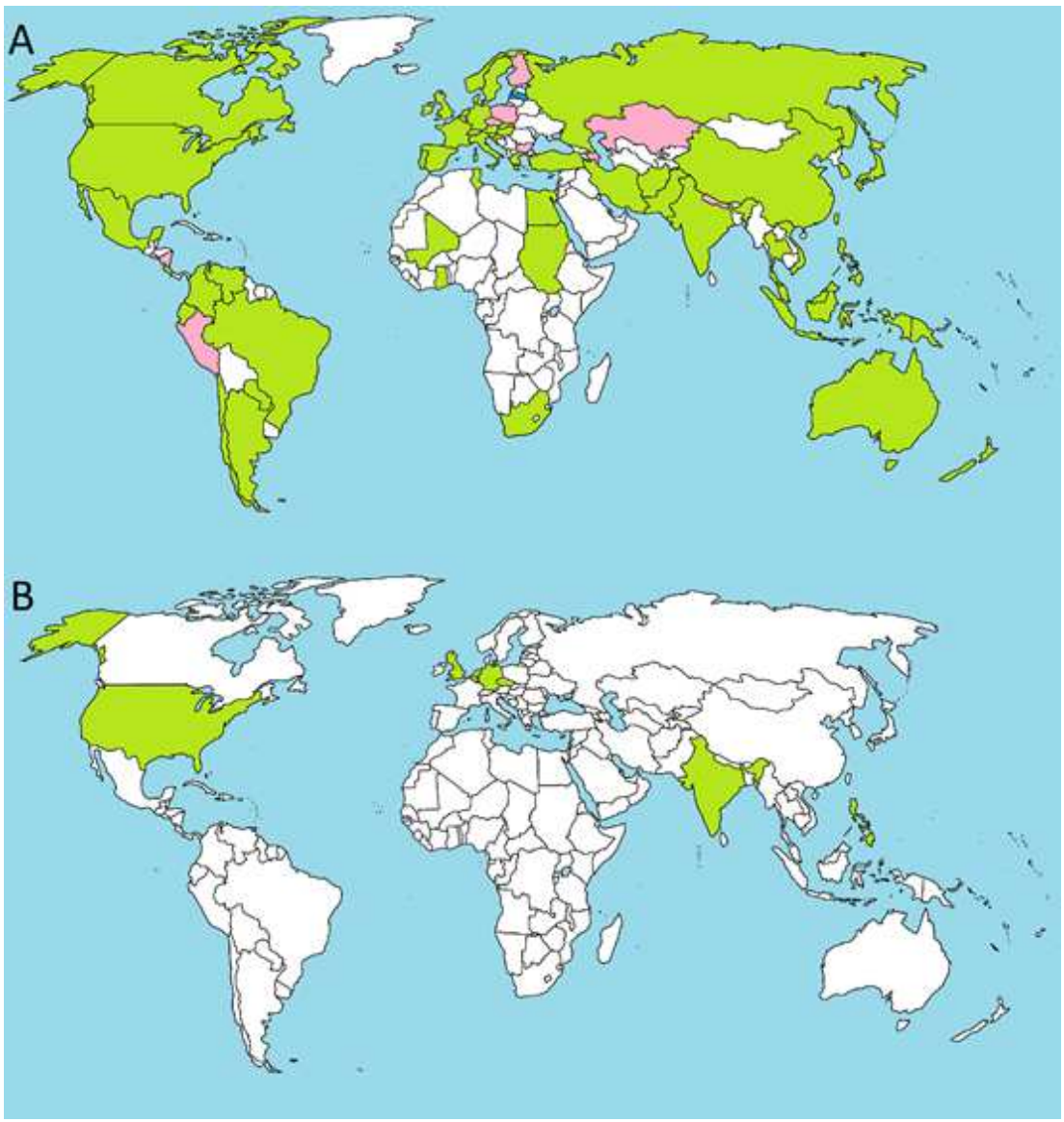


Figure 5. Countries with reported cases of AK are coloured green. The countries where *Acanthamoeba* has been reported, but have no reported cases of AK are coloured pink. A) Shows the countries in which it was possible to find literature with reported presence of *Acanthamoeba* or AK in 2017. B) Shows the countries that had reported cases of AK in 1987 as reviewed by Auran, Starr, & Jakobiec (1987). By 1987, Australia had already a reported case, but it was not included in the article.

1.6.4.3 **Pathogenesis**

AK is a debilitating disease that presents challenges in diagnosis and treatment. Therefore, it is important to understand the pathogenic processes that happen during infection. A better knowledge of the pathogenesis and pathophysiology of AK should lead to better treatments and outcomes for patients (Lorenzo-Morales et al., 2015).

Pathogenic strains regularly show greater thermotolerance, osmotolerance, growth rate, adhesion and immune response evasion (Clarke & Niederkorn, 2006; Lorenzo-Morales et al., 2013; Siddiqui & Khan, 2012b). Pathogenesis of AK consists of three stages: adhesion, secretion of extracellular proteases and phagocytosis with cell death (Lorenzo-Morales et al., 2013).

Adhesion of *Acanthamoeba* to the corneal epithelium is regulated by the mannose binding protein (MBP) which is a mannose-specific lectin (Alizadeh et al., 2007; Garate et al., 2004). The adhesion starts when MBP binds to the mannose glycoproteins of the epithelium of the cornea (Clarke & Niederkorn, 2006; Garate et al., 2006). The number of acanthopodia present in the organism also affects the binding to the epithelium. Pathogenic strains regularly have a significantly higher amount of acanthopodia. Pathogenic amoebae tend to have over 100 acanthopodia, while non-pathogenic rarely have more than 20 (Khan, 2001).

Once the amoeba binds to the epithelium, it starts producing specific proteases for invasion. Proteases play an important role in any *Acanthamoeba* infection, and in AK they start with the degradation of the cornea. *Acanthamoeba* produces three kinds of proteases: serine proteases, cysteine protease and metalloproteases. Pathogenic strains show a different and higher rate of protease activity than non-pathogenic varieties (Panjwani, 2010). It first produces a 133 kDa mannose dependent protease important in the destruction of the epithelium. This protease also exacerbates the release of other cytopathic factors (Leher et al., 1998). Once the amoebae have breached the epithelium, it produces other collagenolytic proteases capable of penetrating and degrading the stroma. It also produces other factors involved in corneal degradation such as elastases, phospholipases and a pore forming protein called acanthaporin (Lorenzo-Morales et al., 2015).

Finally, *Acanthamoeba* phagocytes and produces cell death in the cornea through the action of amoebostomes (Siddiqui & Khan, 2012b). This process results in the destruction of the host cells. Also, *Acanthamoeba* is capable of inducing apoptosis in host cells (Alizadeh et al., 1994; Siddiqui & Khan, 2012b).

After invading the stroma, *Acanthamoeba* continues until reaching the endothelium, where it usually stops. In rare occasions, the infection becomes intraocular (Heffler et al., 1996). A simplified version of the pathogenesis of AK can be found in Figure 6.

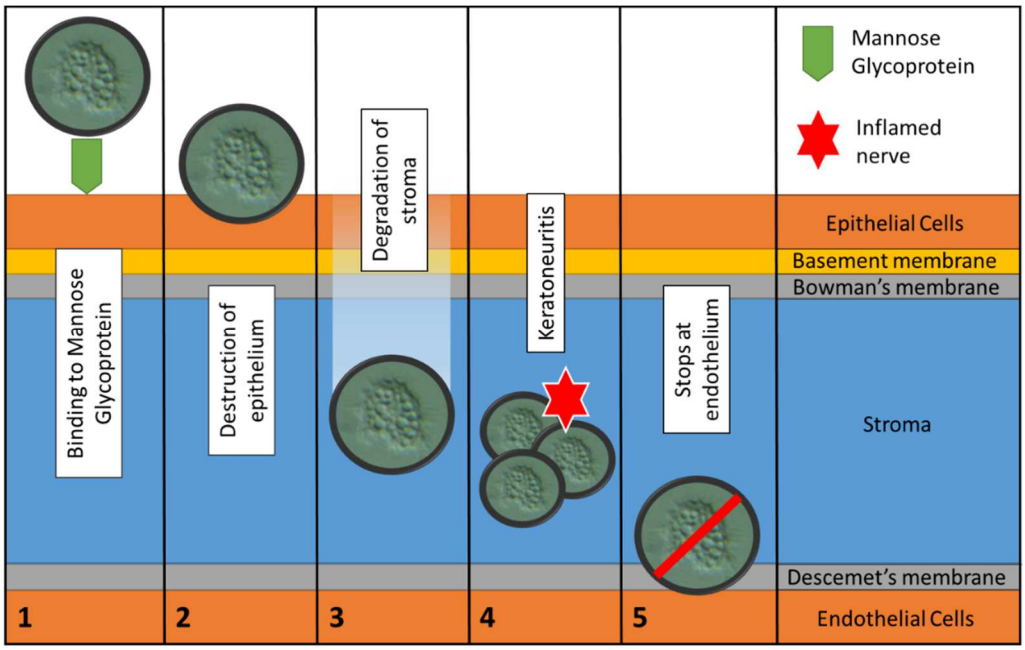


Figure 6. Pathogenesis of AK. 1) *Acanthamoeba* binds to mannose glycoprotein through MBP. 2) Once bound, *Acanthamoeba* produces proteases to destroy the epithelium and enter the cornea. 3) *Acanthamoeba* keeps degrading tissue into the stroma through protease and phagocytosis. 4) Several organisms join around neurons and produce keratoneuritis. 5) *Acanthamoeba* stops before entering the endothelium. Adapted from (Clarke & Niederkorn, 2006; Lorenzo-Morales et al., 2015).

Acanthamoeba cells regularly cluster around corneal nerves causing an inflammatory response of the nerves called keratoneuritis (Clarke & Niederkorn, 2006). The amount of pain of AK patients is not commensurate with the amount of cellular destruction. It is unclear why there is such a painful reaction, as it is not equivalent to other microbial keratitis. It has been speculated that the severe pain is a result of the chemotactic responses from *Acanthamoeba* interaction with nerve cells plus the effect of proteases that produce nerve damage (Clarke & Niederkorn, 2006).

1.6.4.4 Immunity

Since inflammatory response in the cornea leads to impaired vision, this organ has evolved immune privilege, which means it can tolerate a certain amount of antigens without triggering an inflammatory response (Cursiefen, 2007). When infection

occurs, the immune privilege makes dealing with the parasites challenging. *Acanthamoeba* can trigger an adaptive response producing anti-*Acanthamoeba* antibodies. However, these antibodies are incapable of providing immunity inside the cornea (Alizadeh et al., 2001; Neelam & Niederkorn, 2017).

Acanthamoeba is capable of producing an inflammatory response causing some of the symptoms of AK, as the immune response is problematic in the cornea. This response is characterized by macrophages and neutrophils being the primary cell types found surrounding trophozoites or cysts (Marciano-Cabral & Cabral, 2003; Mathers et al., 1987). Macrophages provide protection against *Acanthamoeba* infection in animals (Marciano-Cabral & Cabral, 2003). However, it seems *Acanthamoeba* can hide its antigens preventing macrophage function and lymphocyte response (Mathers et al., 1987). Meanwhile, neutrophils appear to play an important role in preventing *Acanthamoeba* from becoming an intraocular infection and producing endophtalmitis or retinitis (Clarke et al., 2005).

Mucosal immunity in the eye also plays a significant factor against *Acanthamoeba*. However, when eye abrasions occur, the mucosal response includes the upregulations of mannosylated glycoproteins that lead to *Acanthamoeba* adhesion (Neelam & Niederkorn, 2017).

1.6.4.5 Diagnosis

Diagnosis of AK is challenging, and misdiagnosis is common. The symptoms at the beginning stages of AK are very similar to those of viral, bacterial or fungal keratitis. The most commonly accepted means of diagnosis is confocal microscopy, as it is non-invasive and has high-sensitivity. However, confirmation through the cultivation of *Acanthamoeba* is required. Typically, isolation is performed from corneal biopsies or from contact lenses and their storage cases. Also, PCR and immunofluorescence are routinely used (da Rocha-Azevedo et al., 2009; Lorenzo-Morales et al., 2015).

1.6.4.6 Therapy

There is no specific licensed treatment for AK. The recommended treatment by the NHS includes antiseptic drops (chlorhexidine 0.02%, propamidine isetionate 0.1%

and 0.02% polyhexamethylene biguanide) and can be supplemented with anti-inflammatories and painkillers (Hospital, 2017; Titcomb & Jamieson, 2013). However, several other drugs and combinations of them are used. Some of the different drugs used for treatment are shown in Table 5.

Treatment	Reference
Polyhexamethylene Biguanide (PHMB)	(Larkin et al., 1992)
Chlorhexidine with aromatic diamidines	(Kosrirukvongs et al., 1999)
Itraconazole with miconazole and ketoconazole	(Ishibashi et al., 1992)
Variconazole	(Cabello-Vílchez et al., 2014)
Corticosteroids	(Park et al., 1997)
Keratoplasty	(Blackman et al., 1984)

Table 5. List of different drugs used for the treatment of *Acanthamoeba* keratitis.

Other experimental treatments have been studied due to the necessity of finding new and effective treatments. Some of these treatments are shown in Table 6.

Treatment	Reference
Tigecycline	(Jha et al., 2015)
Terbium trinitrate 18-crown ether-6	(Kusrini et al., 2016)
Lippia essential oils	(Gomes de Amorim Santos et al., 2016)
Tea tree oil	(Hadas et al., 2017)
Cellulase inhibitors	(Moon et al., 2015)

Table 6. List of several drugs and chemicals tested for amoebicidal effects in vitro as potential treatments against *Acanthamoeba*.

Since most of the available treatments are not very effective, novel therapeutic approaches are required. The recently published *Acanthamoeba* genome provides potential new approaches to dealing with *Acanthamoeba* infections (Clarke et al., 2013). In Mexico, a successful case received a combination of ultraviolet-A light (UV-A) and riboflavin (Garduño-Vieyra et al., 2011). Khan later confirmed the effect of UV-A light in the treatment of AK (Khan et al., 2011). Additionally, studies of "pharmaceutical phylogeny" are being performed in the hope of finding adequate treatments for AK (Lorenzo-Morales et al., 2016). Another option may be siRNA,

which could help elucidate new pathways and potential new targets for treatment (Martín-Navarro et al., 2013).

Some have identified the cyst as the key towards the development of effective therapy against AK (Lorenzo-Morales et al., 2013). The presence of cyst leads to recurrent infection as they can survive the available treatments (Larkin et al., 1992; Turner et al., 2004).

1.6.4.7 Impact

In the United States, there are around 930,000 visits for keratitis and contact lens disorders, of which 58,000 are emergencies. In 2010, these cases had an estimated cost of $175 million dollars in direct health care expenditure (Cope et al., 2015).

Unlike other eye infections, the quality of life of AK patients is negatively affected. In a survey, patients consider that their overall physical health and their social functioning were affected by the infection (Carnt et al., 2015).

1.7 Objectives of the project

Acanthamoeba is a facultative pathogen found worldwide that has been steadily growing in importance. Therefore, the understanding of its biology and taxonomy is paramount when dealing with the amoebae, whether it is as a disease agent or as a member of the ecosystem. As the incidence of AK and bacterial infections related to *Acanthamoeba* rises, the importance of understanding the different aspects of *Acanthamoeba* biology become crucial.

The project started with the objective of identifying potential antimicrobials from *Acanthamoeba*. By the end, this objective had branched out into three main goals. First, isolate and identify new strains of *Acanthamoeba* and new species of amoebae. It is important to work with recently isolated strains that keep the characteristics shown in the environment.

Second, identify factors involved in *Acanthamoeba* encystment. One of the keys towards combating AK has been identified as the encystment, as it is the main reason

for the high persistence of infections (Lorenzo-Morales et al., 2015). A deeper understanding of the encystation process might allow developing new and more effective antimicrobials against AK.

Finally, identify mechanisms through which *Acanthamoeba* feeds on bacteria for the characterisation of antimicrobial proteins. The close relationship between *Acanthamoeba* and some bacteria provides opportunities to identify factors and potential options for dealing with several pathogenic agents. In addition, focusing on eukaryotes as a source of bactericidal agents lowers the risk of lateral transfer and development of resistance.

Chapter 2 Materials and Methods

2.1 Culture media

2.1.1 Neff's saline

A modified version of Neff's saline (NS) was used for *Acanthamoeba* isolation (Page, 1967). It consisted of NaCl (0.60 g/5 L), $MgSO_4.7H_2O$ (0.02 g/5 L), $CaCl_2.2H_2O$ (0.02 g/5 L), Na_2HPO_4 (0.71 g/5 L) and KH_2PO_4 (0.68 g/5 L).

2.1.2 AX2 media

AX2 media was used for *Acanthamoeba* axenic culture. It consisted of Bacto trytone (14.3 g/L), yeast extract (7.15 g/L), glucose (15.4 g/L), Na_2HPO_4 (0.51 g/L) and KH_2PO_4 (0.486 g/L) with a pH of 6.5. This media was first used to culture *Dictyostelium discoideum* Ax-2 amoeba (Watts & Ashworth, 1970).

2.1.3 Lysogeny broth

Lysogeny broth (LB media) was used to culture *E. coli*. It was prepared using Invitrogen's Luria Broth Base following manufacturer's instructions.

2.1.4 Vandamme media

Vandamme media (VD media) was used to culture *Arcobacter butzlerii*. It consisted of special peptone (10 g/L), Lab Lemco powder (5 g/L), yeast extract (5 g/L), NaCl (5 g/L), sodium succinate.$6H_2O$ (3.25 g/L), L-Glutamic acid-Na salt (2 g/L) and $MgSO_4.7H_2O$ (2.01 g/L) with a pH 6.5.

2.1.5 2xTY media

2xTY media was used for cultivation of transformed *E. coli*. It consisted of Bacto tryptone (10 g/L), yeast extract (5 g/L) and NaCl (10 g/L).

2.1.6 Terrific broth

Terrific broth was used for cultivation of transformed *E. coli*. It consisted of Bacto tryptone (12 g/L), yeast extract (24 g/L), glycerol (4 ml/L) in 900 ml ddH_2O and autoclaved. Also, 0.17 M KH_2PO_4, 0.72 M K_2HPO_4 (2.31 g/L and 12.54 g/L respectively) in 100 ml of ddH_2O were autoclaved. Once the temperature of the solution was below 60°C, they were mixed. Terrific broth mix (Melford) was also used for the preparation of TB media.

2.1.7 Neff's encystment media

Neff's encystment media (NEM) was used to induce encystment (Neff et al., 1964b). It consisted of 0.1 M KCl, 8 mM $MgSO_4$, 0.4 mM $CaCl_2$, 1 mM $NaHCO_3$, 20 mM 2-amino-2-methyl-1,3-propanediol, pH 8.8. I. 2-amino-2-methyl-1,3-propanediol could be substituted with 10 mM Tris-HCl pH 8.0 with similar results.

2.1.8 Agar plates

Agar plates were prepared from the different media as needed using 1.5% agar (Sigma-Aldrich).

2.1.9 Antibiotics

Antibiotics were used when required by the experiment. The antibiotics and the concentration used in the experiments are shown in Table 7. All antibiotics were supplied by Sigma-Aldrich.

Antibiotic	Conc. (µg/ml)	Application
Ampicillin	100	Amoebae isolation and aLICator cloning
Carbenicillin	50	Amoebae isolation and aLICator cloning
Chloramphenicol	35	pLysS cells and Gateway cloning
Kanamycin	50	Gateway cloning

Table 7. Antibiotics used and their concentrations.

2.2 Bacterial Cultures

2.2.1 *Escherichia coli*

E. coli were grown overnight in LB, 2xTY or TB depending on the purpose. Cultures were incubated at 37°C in an orbital shaker at 200 rpm.

2.2.2 *Arcobacter butzlerii*

Arcobacter butzlerii were grown for 4 days at 30°C with VD media in microaerophilic culture done with a seal steel chamber and candles. Afterwards, cultures were grown at 30°C.

2.2.3 Bacteria cryopreservation

Bacteria cultures were preserved in 20% glycerol and stored at -80°C. This was performed after growing a bacterial culture overnight. Later, cultures were grown until reaching logarithmic phase. The bacteria were centrifuged and the supernatant discarded. Fresh media with glycerol was added to the bacterial pellet and transferred to a cryogenic tube. The tubes were stored at -20°C overnight, before being transferred to -80°C. For continuous use, bacteria grown in liquid media were streaked in LB plates and stored overnight at 37°C. Plates were stored at 4°C. When new cultures were needed, a colony was selected and grown in liquid media in a rotating incubator at 37°C.

2.3 Amoebae cultures

Unless otherwise stated, amoebae cultures were grown at room temperature which was considered to be between 20 and 25˚C.

2.3.1 Amoebae isolation

Amoebae isolation comes from environmental sampling and the different steps to obtain axenized cultures or co-cultures of a single strain of amoebae with *E. coli*.

2.3.1.1 Environmental sampling

Samples with organic material were collected from different environments. These environments comprised terrestrial and aquatic, which included lakes, rivers and sea. Samples were collected in Eppendorf tubes.

2.3.1.2 *Acanthamoeba* isolation

Environmental samples were placed in plates containing NS agar covered with liquid NS. Plates were monitored using a microscope for the presence of amoebae. Once amoebae were observed, agar pieces were cut and placed in new plates seeded with *E. coli.* This step was repeated three to four times. After amoebae were observed, a new piece of agar was cut and passed to a new plate seeded with inactivated *E. coli* (60°C for 2 h). This step was repeated at least 3 times. Finally, a small piece of agar was cut and placed on a plate with AX2 media agar with ampicillin or carbenicillin. If the plate did not show contamination and amoebic growth was visible with a microscope, small pieces of agar were passed to liquid AX2 media with antibiotics. Finally, the culture was prepared for cryopreservation and long-term storage. A simplified diagram of the process is shown in Figure 7.

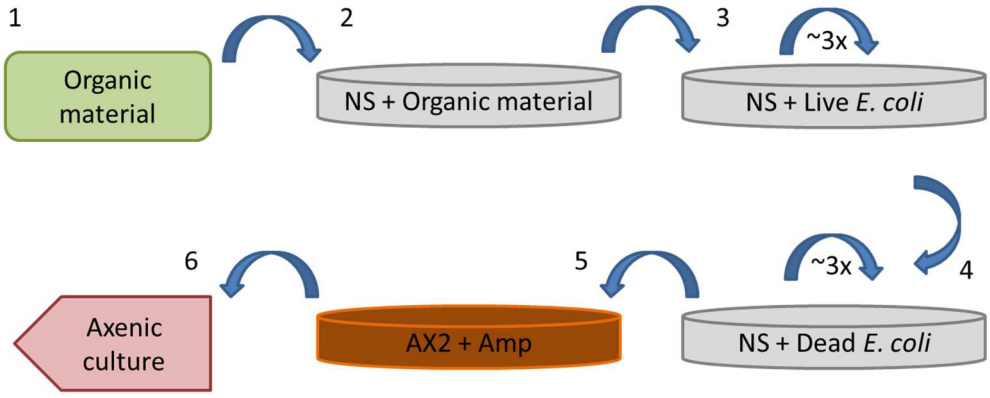


Figure 7. Simplified diagram showing the process of *Acanthamoeba* isolation, from organic material to axenic culture.

2.3.1.3 Isolation of amoeba different from *Acanthamoeba*.

Environmental samples were placed on a plate containing NS agar covered with liquid NS. Plates were monitored using a microscope for the presence of amoebae. Once amoebae were observed, agar pieces were cut and placed in new plates seeded with *E. coli*. After amoebae were observed in the new plate, another piece of agar was cut and passed to a new plate seeded with *E. coli*. After several passes, when only one type of amoebae was visible, the amoebae were passed to 25 cm^2 flasks with NS and *E. coli*. Later, the co-culture of amoebae and *E. coli* was prepared for cryopreservation for long-term storage. A simplified diagram of the process is shown in Figure 8.

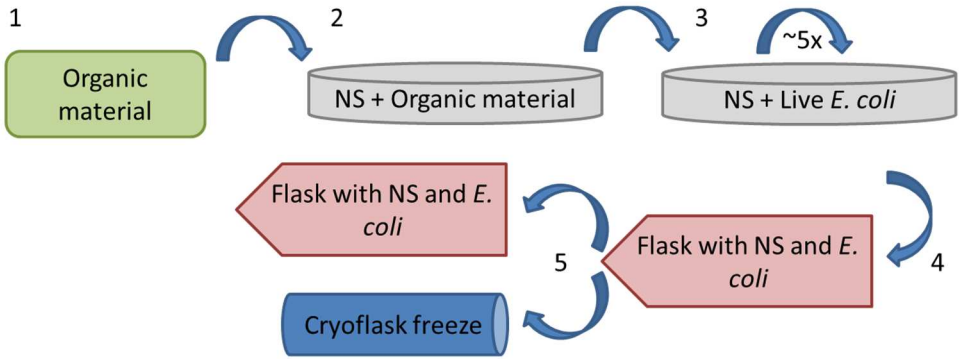


Figure 8. Simplified diagram showing the process of amoebae isolation in co-culture with *E. coli*.

2.3.1.4 Axenized *Acanthamoeba* cultures

Acanthamoeba strains were grown with AX2 media at room temperature in T25 or T75 (25 or 75 cm^2) Corning flasks depending on the experiment. For experiments where a high concentration of amoebae was required, Erlenmeyer flasks were used at 25° C with rotation. The propagation of cultures was done by serial passage.

2.3.1.5 Amoebae and *E. coli* co-cultures

Isolated amoeba incapable of axenic growth, were cultivated in NS agar plates with *E. coli* lawns or in NS with suspended *E. coli* in T25 Corning flasks at room temperature. The propagation of cultures was done by serial passage.

2.3.1.6 Amoebae cryopreservation

Amoebae cultures were preserved in the proper amoebae media (NS for amoebae and *E. coli* co-cultures or AX2 for axenic cultures) with 10% DMSO and stored in cryogenic tubes at -80°C. First, the cultures were centrifuged and suspended in media. Later, the tubes were stored at room temperature for 2 h before being incubated at -20°C overnight. Finally, the cultures were stored at -80°C.

2.4 Amoebae characterisation assays

2.4.1 Osmolarity tolerance assay

When appropriate, isolated *Acanthamoeba* osmolarity tolerance was tested. *Acanthamoeba* was grown on NS agar plates with 0.5 M, 1 M and 1.5 M mannitol and seeded with *E. coli* lawns as previously described (Khan et al., 2001).

2.4.2 Thermotolerance assay

When appropriate, isolated *Acanthamoeba* thermotolerance was tested. *Acanthamoeba* cultures were grown on NS agar plates seeded with *E. coli* lawns and incubated at different temperatures ranging from 25°C to 46°C as previously described (Khan et al., 2001).

2.4.3 Cyst thermotolerance

When appropriate, isolated *Acanthamoeba* cysts' thermotolerance was tested. Cultures were incubated in NEM until most cells had encysted. The cultures were centrifuged and the supernatant discarded. The cysts were resuspended in NS and incubated at 37°C, 45°C, 55°C, 60°C and 65°C for 10 min. The cysts were transferred to NS plates seeded with *E. coli* lawns. Cultures were monitored periodically for trophozoites.

2.4.4 Cell viability by Trypan blue exclusion

The viability of *Acanthamoeba* cells was determined by adding equal volumes (50 µl) of 0.4% Trypan blue (from Sigma-Aldrich) and *Acanthamoeba* suspension. 10 µl of the suspension were loaded into a haemocytometer. Dead cells take up the dye while live cells do not. Unstained and blue-stained cells in the grid were counted, and the concentration of cells was calculated considering the dilution factor (=2) and the correction factor (10^4). The percentage of viable cells was determined by dividing the number of viable cells by the total number and multiplied by 100.

2.5 Microscopy

2.5.1 Optical microscopy

Cultures and cells were observed and monitored using a Leica DM IRB inverted microscope. The microscope was plugged into a Canon EOS 1100D camera. The EOS utility software was used to record photographic evidence.

2.5.2 Time-lapse video microscopy

Pictures were taken at regular intervals from amoebae and different stages to show encystment, locomotion, aggregation of cysts, etc. Time-lapse videos were created using the software VirtualDub using different conditions depending on the number of pictures and length of the video (Lee, 2005).

2.6 Molecular Biology

2.6.1 Axenized *Acanthamoeba* DNA extraction (Lorenzo-Morales et al., 2005a)

The DNA extraction from *Acanthamoeba* was performed following a modified phenol-chloroform method. *Acanthamoeba* cultures were grown in a 25 cm^2 culture flask to near confluence. The monolayer was disrupted so that cells were floating in the

media. A sample was taken and located in an Eppendorf tube. The sample was centrifuged for 10 min at 150 g and the supernatant discarded. The pellet was washed by resuspending it in NS and centrifuged again. The pellet was resuspended in 500 µl lysis buffer (30 mM Tris-HCL pH 7.4, 5 mM EDTA, 100 mM NaCl pH 8.0, and 1% SDS) with 10 µl of proteinase K. The sample was incubated for 2 h at 60°C. Afterwards, the culture was heated to 95°C for 10 min to stop proteinase K activity. 500 µl of buffered phenol were added and mixed gently. The sample was centrifuged for 10 min at 110000 g. The aqueous phase was transferred to a new tube. If the sample was cloudy, the sample could be treated again with phenol up to three times. Once the aqueous phase was transferred to a clean tube, 500 µl of chloroform were added and mixed gently. The sample was centrifuged for 10 min at 10000 g. The aqueous phase was transferred to a new tube where 500 µl of ice-cold Isopropanol and 100 µl of 3 M sodium acetate were added. The sample was mixed gently and incubated at -20°C overnight. The sample was centrifuged for 20 min at 10000 g, and the supernatant was discarded. The sample was left to dry and the pellet resuspended in 30 µl of ddH_20. The DNA concentration was measured using a NanoDrop 2000 (Thermo Fisher Scientific).

2.6.2 DNA extraction from not axenic amoebae (modified from Lorenzo-Morales et al., 2005)

DNA extraction from co-cultures of amoebae and bacteria or yeast were performed with phenol chloroform method. The protocol was similar to DNA extraction from axenized *Acanthamoeba*, but the collection of the amoebae differed. Cells were scraped from agar plates seeded with *E. coli* and collected into an Eppendorf tube. A larger volume of sample was collected and centrifuged at a faster rate over a larger period of time (1500 g for at least 20 min or until the pellet formed) than axenic cultures, until a pellet was formed. Samples were washed with NS before adding the lysis buffer. The extraction was performed using the previously described phenol - chloroform method.

2.6.3 PCR for 18S rDNA amplification

The 18S rDNA fragment was amplified by PCR for the identification of the isolated amoebae. The regular set of primers used were called Euk18S (Corsaro et al., 2010).

These primers amplified using an annealing time of 55˚C and an extension time of 2 min. When the fragment did not amplify, other sets of primers that gave smaller fragments were used. JDP1 and JDP2 amplify the ASA.S1 diagnostic fragment (around 500 bp) and the CRN5 with the 1137 primers amplify the GTSA.B1 (around 1400 bp). The ASA.S1 and full 18S gene were amplified when the strain showed morphological characteristics representative of *Acanthamoeba*. When the amoebae morphology showed a different genus from *Acanthamoeba*, the 18S rDNA was amplified using the same primers, but the smaller fragments were ignored. The PCR was performed with GoTaq Green Master mix (Promega) using 50 µl reactions. All primers were obtained from Sigma-Aldrich.

Primer	Fragment	Species	Approx. Size	Sequence
JDP1	ASA.S1 F	*Acanthamoeba*	500	GGCCCAGATCGTTTACCGTGAA
JDP2	ASA.S1 R	*Acanthamoeba*	500	TGACTCCCCTAGCAGCTTGTGAGA
CRN5	GTSA.B1 F	*Acanthamoeba*	1500	CTGGTTGATCCTGCCAGTAG
1137	GTSA.B1 R	*Acanthamoeba*	1500	ATTGACGGAAGGGCAC
Euk18sF	18S rDNA	All amoebae	2000	GACTGGTTGATCCTGCCAG
Euk18sR	18S rDNA	All amoebae	2000	TGATCCTTTCGCAGGTTCAC

Table 8. Primers for the amplification of 18S rDNA (Corsaro et al., 2015; Dyková et al., 1999; Schroeder et al., 2001; Weekers et al., 1994).

When amplifying genes from co-cultures with amoebae and yeast, the gel had to be run for a longer period of time to separate amplicons with similar size in case the fragments from both organisms were amplified. If two amplicons were amplified, the bands had to be excised and purified.

2.6.4 Bacterial endosymbionts identification

When bacterial endosymbionts were observed, the identification of the bacteria was performed by sequencing. DNA was extracted from amoebae culture, and PCR was performed to amplify the 16S rDNA fragment.

The PCR reactions were performed using GoTaq Green Master Mix. The initial denaturation was done with 95°C for 5 min, followed by 35 cycles of 1 min 95°C

denaturation, 45 s 58°C extension, 2 min of 72°C elongation, finalizing with 7 min of 72°C final elongation.

2.6.5 DNA electrophoresis

Electrophoresis was run on a 1% agarose gel at 80 V with TAE buffer (Unless a different concentration was required). Gels were stained using Sybr-Safe (Sigma-Aldrich). Track-it 100 bp ladder (Invitrogen) was used to determine the size of the fragments.

2.6.6 PCR fragment purification

PCR fragments were purified using QIAquick PCR Purification Kit or QIAquick Gel Extraction Kit (QIAGEN) following manufacturer's instructions.

2.6.7 DNA sequencing

DNA sequencing was carried out by Edinburgh Genomics at The University of Edinburgh. It was performed using ABI 3730 capillary sequencing instruments. The sequencing was performed using the original PCR primers. When longer 18S rDNA amplicons were amplified, the primer 1137 was used to sequence the mid-part of the amplicon.

2.7 Proteins

2.7.1 Protein precipitation

Proteins were precipitated from *Acanthamoeba* culture supernatant by growing 500 ml cultures and centrifugation to separate the media from the amoebae. Proteins were precipitated using 46% ammonium sulphate, which was added slowly to the sample while stirring. The solution was incubated with light stirring overnight at 4˚C. The sample was centrifuged at 3000 g for 15 min at 4˚C. The supernatant was discarded and the precipitated proteins resuspended in ddH_2O.

2.7.2 Protein dialysis

After precipitation of the proteins, the samples were dialysed using Visking dialysis tubing size no. 1 (Medicell). Before loading the sample, the membrane was hydrated with ddH_2O. Later, the protein was loaded into the membrane, and left overnight in 20 mM Tris buffer at 4˚C.

2.7.3 Ion chromatography

Chromatography was performed on separate excreted proteins from *Acanthamoeba*. The chromatography was performed using CM Sepharose Cation Exchanger or DEAE Sepharose Anion Exchanger (Sigma-Aldrich).

A column was loaded with the suitable resin and equilibrated with buffer 0.5 M Tris at the desired pH overnight. Then, the column was equilibrated by running 1 L of 50 mM Tris buffer. The sample with the solubilized proteins was loaded into the column. The fractions were collected starting at this step. Once the sample settled, it was washed with four volumes of 50 mM Tris buffer. The column was eluted using a gradient solution starting with washing buffer and gradually increasing to 0.5 M KCl. Finally, the column was washed with 1 M KCl buffer.

2.7.4 SDS-PAGE protein electrophoresis

Using a Bio-Rad “mini-Protean” system, SDS-PAGE electrophoresis was run using 10% acrylamide resolving gels that consisted in ddH_2O (4.33 ml), 1.5 M Tris pH 8.8 (2.5 ml), 30% acrylamide (3 ml), 10% SDS (100 µl), 10% APS (50 µl) and TEMED (20 µl). The stacking gel consisted of ddH_2O (6.1 ml), 0.5 M Tris-HCL pH 6.8 (2.5 ml), 10% SDS (100 µl), 30% acrylamide (1.3 ml), 10% APS (50 µl) and TEMED (20 µl).

Electrophoretic separation was carried out in electrode buffer (5X buffer consists of Tris base 15 g/L, Glycine 72 g/L and SDS 5 g/L). The sample was mixed with sample buffer (2X buffer consists of 1 ml of 1 M Tris pH 6.8, 2 ml glycerol, 2.5 ml β-mercaptoethanol, 4 ml 10% SDS and 500 µl 1% bromophenol blue). Electrophoretic separation was carried out at 200 V until the sample dye reached the bottom of the gel. Unstained protein MW markers (Thermo Fisher Scientific) was used. Gels were

stained using Coomasie blue for 1 h and distained with distaining solution (containing 10% acetic acid and 5% methanol).

2.7.5 Protease zymogram

Protease zymograms were run using similar conditions to SDS-PAGE gels, with the addition of 0.1% fish gelatine (from Sigma-Aldrich) to the resolving gel. Gels were incubated gel with 2.5% Triton X-100; 50 mM Tris-HCl pH 7.0 for 1 h and then overnight in 50 mM Tris-HCl pH 7.0, 2 mM $CaCl_2$ at room temperature with continuous shaking. Gels were rinsed and stained with Coomasie stain. After one hour, the gels were distained with distaining solution.

2.7.6 Protease inhibitors

Protease inhibitors where used before running the protease zymogram to classify the proteases. The following protease inhibitors: phenylmethylsulfonyl fluoride (PMSF), EDTA (not an actual protease inhibitor, but a metal ion chelator that can inhibit some proteases) and benzamidine.

2.7.7 Antimicrobial zymogram

Overnight cultures of *E. coli* or *A. butzlerii* were grown in their respective media. 6 ml of bacterial culture were centrifuged (10000 g for 2 min). The supernatant was discarded. The remaining bacteria were resuspended in 1 ml of ddH_2O. An SDS-PAGE gel was prepared, but 1 ml of ddH_2O was substituted with the bacterial mix. Electrophoretic separation was carried out at 200 V at 4˚C until the sample dye reached the bottom of the gel. Gels were incubated gel with 2.5% Triton X-100; 50 mM Tris-HCl pH 7.0 for 1 h and then overnight at in 50 mM Tris-HCl pH 7.0, 2 mM $CaCl_2$ at room temperature with continuous shaking. Gels were rinsed and stained with Coomasie stain and distained with distaining solution just like in the SDS PAGE method.

2.7.8 Cellulase zymogram (modified from Willis, Klingeman, Oppert, Oppert, & Jurat-Fuentes, 2010)

Cellulase zymograms were run using similar conditions to SDS-PAGE gels, with the addition of 0.2% carboxymethyl cellulose (from Sigma-Aldrich) to the resolving gel. Electrophoretic separation was carried out at 100 V at 4˚C until the sample dye reached the bottom of the gel. Gels were washed 5 times for 6 minutes in 50 ml of wash buffer containing 0.1 M sodium succinate, 20 mM β-mercaptoethanol, pH 5.8. Gels were incubated in wash buffer for 30 min and stained with Congo red for 10 to 15 min. Gels were distained with 1 M NaCl solution.

2.7.9 Amylases zymogram (modified from Upadhyay, Sharma, Pandey, & Rajak, 2005)

Amylase zymograms were run using similar conditions to SDS-PAGE gels, with the addition of 1.5 mg/ml of starch to the resolving gel. Electrophoretic separation was carried out at 100 V at 4˚C until the sample dye reached the bottom of the gel. Gels were washed for 10 min with buffer containing 2% Triton X-100, in 50 mM Tris pH 7.4 and slight agitation. Gels were washed for 10 min with buffer containing 50 mM Tris pH 7.4. Gels were incubated for 3 h in substrate buffer containing 0.3 g $CaCl_2$, 0.01 g NaCl, 0.3 g NaN_3, 0.5 ml Triton X-100 in 50 ml of 50 mM Tris buffer pH 5.5. Gels were stained with lugol iodine (50 ml were prepared with 0.1 g KI and 005 g I_2) for 5 min. Gels were rinsed and distained with H_2O.

2.7.10 Pectinase zymogram (modified from van Dyk, Sakka, Sakka, & Pletschke, 2010)

Pectinase zymograms were run using similar conditions to SDS-PAGE gels, with the addition of 0.1% pectin (from Sigma-Aldrich) to the resolving gel. Electrophoretic separation was carried out at 100 V at 4˚C until the sample dye reached the bottom of the gel. Gels were washed for 10 min with buffer containing 2.5% Triton X-100, in 50 mM Tris pH 7.4 and slight agitation. Gels were washed for 12 h with buffer containing 50 mM Tris pH 7.4 and 0.1 mM to 0.5 mM $CaCl_2$ at 37˚C. Gels were stained using 0.05% Ruthenium Red for 1 h, rinsed and distained with water.

2.8 Cloning of *Acanthamoeba* proteins

Cloning was performed with two different cloning systems following manufacturer's instructions: Gateway Cloning (supplied by Thermo Fisher Scientific) and aLICator Cloning and Expression System (supplied by Thermo Fisher Scientific). Two proteins were selected for cloning in different cloning systems: a protease and a lysozyme.

The simplified cloning process for both systems is presented in Figure 9.

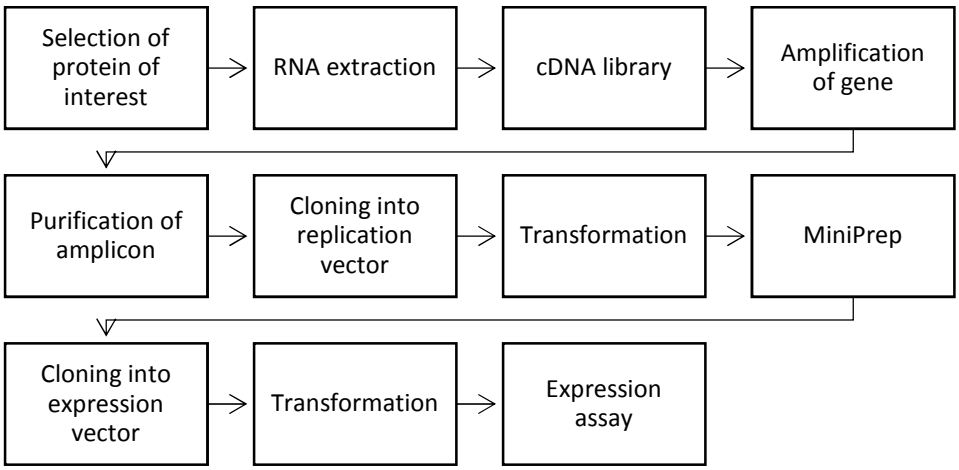


Figure 9. Activities diagram for cloning.

2.8.1 RNA extraction

Extraction of RNA was performed with RNeasy Mini Kit (QIAGEN) following manufacturer's instructions. The RNA was extracted under different circumstances depending on the purpose of the experiment.

2.8.2 Reverse transcription polymerase chain reaction (RT-PCR) for cDNA libraries

RT-PCR was performed to obtain cDNA from total RNA obtained from amoebic cultures. RNA was incubated with OligoDTs at 70˚C for 5 min. Afterward, m-MLV Reverse transcriptase was used at 42˚C for 1 h. cDNA was used for amplification of different genes with standard PCR. Actin was used as a positive control. All reagents were supplied by Promega.

2.8.3 aLICator cloning and expression system

The aLICator Cloning and Expression System (Thermo Fisher Scientific) was used with the pLATE52 plasmid according to manufacturer's instructions. The gene cloned in the vector was the 33 kDa protease referred to as Protease 33. This plasmid was selected because it carries a 6xHis Tag and a cleavage site denominated as WELQut to separate the protein from the 6xHis-tag. A map of the construction built for the protease is found in Figure 10.

The transformation was performed using *E. coli* BL21 DE3 strain and BL21 Rosetta II pLysS strain.

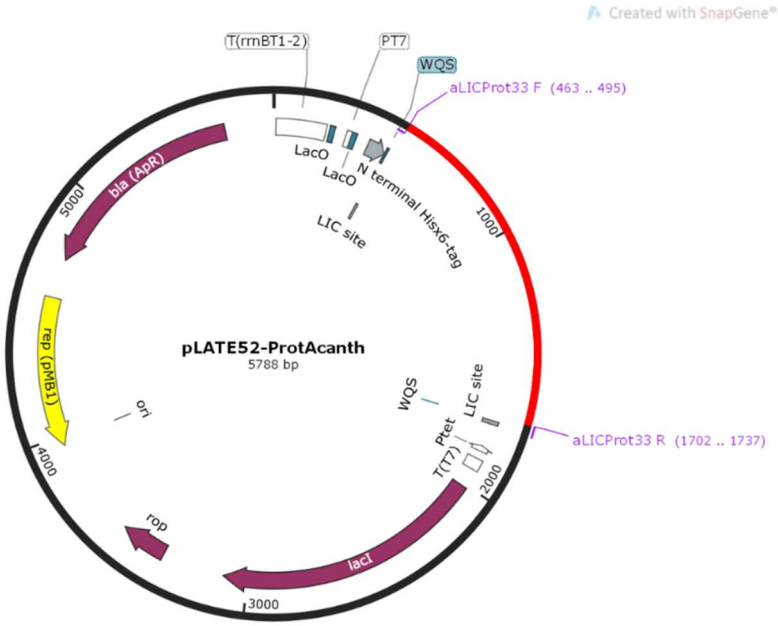


Figure 10. Map of the construction of Protease 33 using pLATE52. Map created for aLICator system where the red line represents the inserted fragment using SnapGene.

2.8.4 Gateway system

Gateway System (Thermo Fisher Scientific) was used for cloning. The genes cloned into the vectors were Protease 33 and the *Acanthamoeba* lysozyme. Primers were designed to include the proper attb1 and attb2 sites. First, the amplicon was cloned into the pDONR221 plasmid for sequencing and storage. Transformation using this plasmid was made into DH5α strain. The cloning of the fragment into the pDONR221

generated the plasmids pENTR+Prot33 (for the protease) and pENTR+Lys (for the lysozyme).

Once the pENTR+Prot33 and pENTR+Lys were obtained and sequenced, it was cloned with the LR reaction of the Gateway system into the pDEST17 (6xHis-tag), and a laboratory assembled plasmid denominated pDEST-MBP (6xHis-tag plus Maltose Binding Protein). Afterward, the pDEST17+Prot33 and pDESTMBP+Prot33 plasmids were transformed into DH5α *E. coli* strains following manufacturer's instructions. The plasmid was extracted, and cloned into *E. coli* BL21 DE3 strain and BL21 Rosetta II pLysS strains for expression. Maps for the constructions with the pENTR and pDEST17 vectors and the proteins can be found in Figure 11 and Figure 12.

2.8.5 Competent cells

Preparation of competent cells was performed following the calcium chloride method. *E. coli* culture was grown overnight. 10 ml of fresh media were inoculated the following day and incubated at 37 ˚C in a rotating incubator until it reached logarithmic phase (OD 600 nm between 0.5 and 1 absorbance). The culture was incubated on ice for 10 min and centrifuged at 150 g for 5 min at 4˚C. The cells were gently resuspended in half the original volume of ice-cold 100 mM $Ca^{2+}Cl_2$ and incubated on ice for 40 min. The sample was centrifuged again, and the pellet was resuspended in 10% of the original volume of 100 mM $Ca^{2+}Cl_2$. For long-term storage of the competent cells, 50% of glycerol was added to the solution of $Ca^{2+}Cl_2$, and the samples were stored at -80˚C.

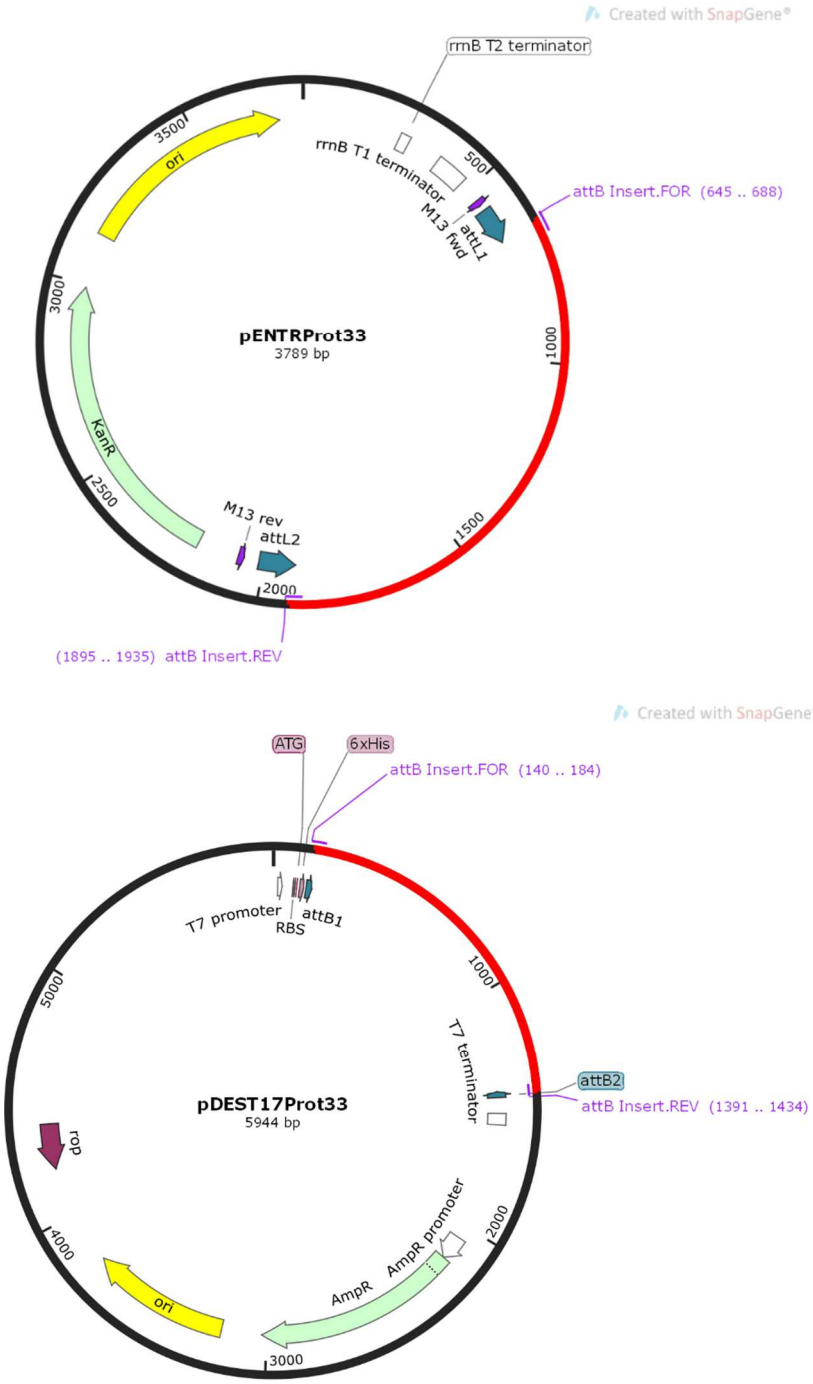


Figure 11. Maps of the constructions of the ENTRY and Expression vectors for Protease 33 using Gateway system. The red line represents the inserted fragment. Images created using SnapGene.

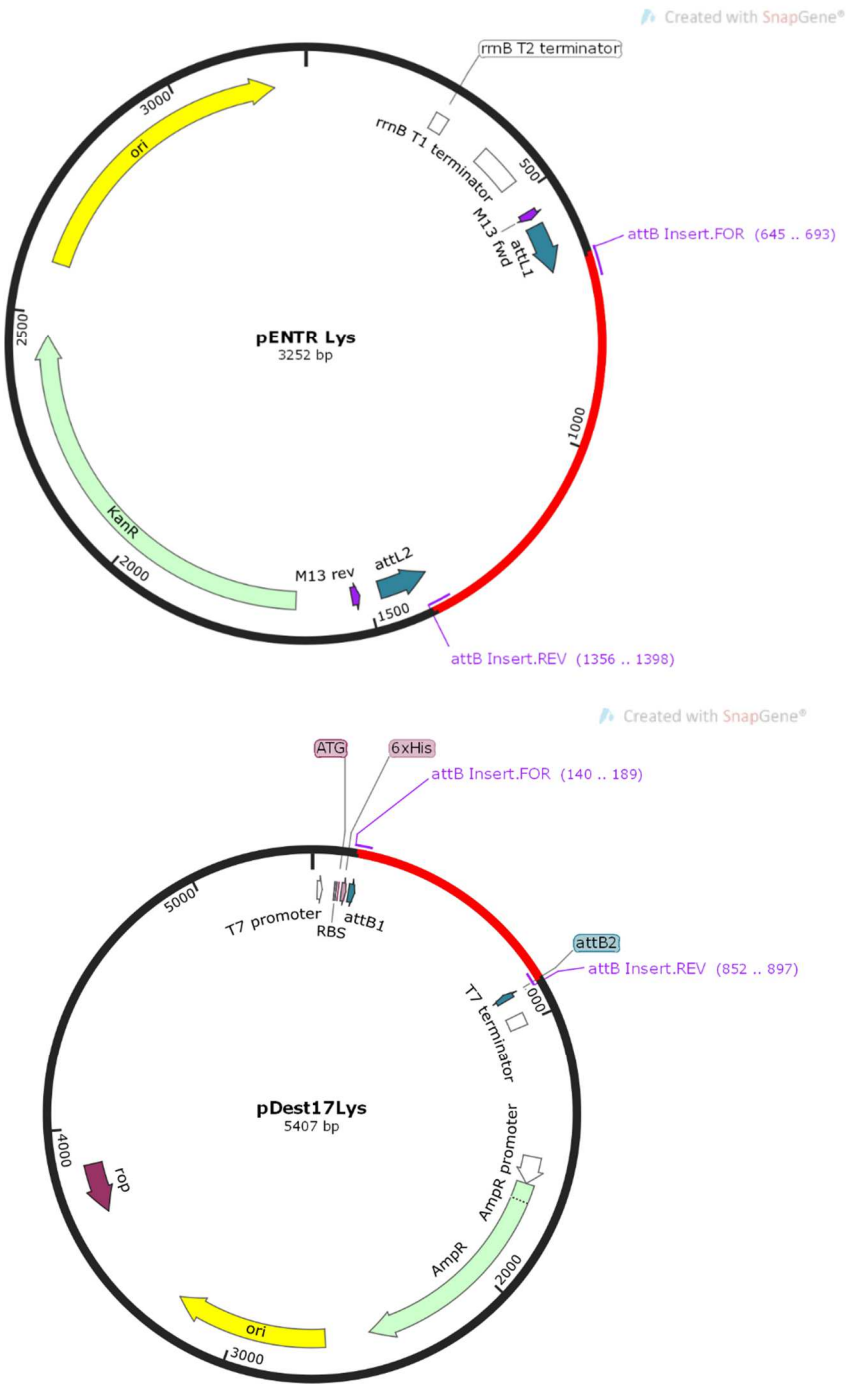


Figure 12. Maps of the construction of the ENTRY and Expression vectors for *Acanthamoeba* lysozyme using Gateway system. The red line represents the inserted fragment. Images created using SnapGene.

2.8.6 Transformation

The plasmid was mixed with 100 µl of competent cells. The cells were incubated for 30 min on ice and heat-shocked for 1 min at 42˚C. The cells were returned to the ice for one minute. One volume of growth media (2xTY or LB media) was added to the cells and incubated at 37˚C for 2 h. The culture was spread on LB agar plates with the proper antibiotic and left to grow overnight at 37˚C.

2.8.7 Plasmid extraction

Plasmid extraction was performed from overnight cultures. The extraction was performed using the QIAprep Spin Miniprep kit (QIAGEN) following manufacturer's instructions.

2.8.8 Colony PCR

Every time a transformation was performed, several colonies were selected. The colonies were picked and dissolved in 50 µl of ddH_20. 45 µl were taken and put in bijou tubes with 2 ml of culture media and the selection antibiotic. The reaction was performed similar to a standard PCR, substituting the DNA with the 5 µl of the leftover dissolved colony, and with a 10 min denaturation cycle at the beginning to break down the bacteria. The reaction was performed in 50 µl reactions using GoTaq Green Master Mix.

2.9 Encystment experiments

2.9.1 Encystment induction

Encystment was induced using NEM (Neff et al., 1964b). Media from confluent cultures was discarded before washing the monolayer with PBS. Enough NEM to cover the culture was added. The cultures were incubated at room temperature.

2.9.2 Encystment rate experiments

NEM was added to different cultures to compare their encystment rates. At different time points, pictures were taken in triplicates. Trophozoites and cysts in the pictures were counted, and an “encystment curve” for the different strains was created.

2.9.3 Conditioned media (CM) for encystment

NEM media was used to encyst cultures of *Acanthamoeba*. After four days, the cultures were collected and centrifuged. The supernatant was collected and used as the conditioned media.

2.9.4 Microscope cell counts during encystment

Encystment dynamics experiments were performed taking pictures at differing time points with different treatments. Afterwards, the cells were counted differentiating trophozoites from cysts. All rounded cells were considered cysts regardless of their stage of encystment.

The strains used for the different experiments included Neff, Neff 42 cycles (42C), Neff 47 cycles (47C), 53, 61 and 64. 42C and 47C are strains of Neff where viable cysts have been selected with SDS with the number indicating how many cycles the strain has been subjected to.

2.9.5 Spectrophotometric measurements for encystment

Samples were stained with Congo red to compare the encystment dynamics of three different treatments including trophozoites in AX2, NEM and conditioned media during four days. 1.5 ml of each sample were centrifuged and incubated with 100 µl of 1 mM Congo red for 10 min. The samples were washed with NS on two occasions. The samples were centrifuged and resuspended in 1 ml of NS. The samples were measured using a spectrophotometer at a wavelength of 495 nm.

2.9.6 Spectrofluorometric measurements for encystment

Katy McCalister performed all spectrofluorometry experiments as part of her dissertation which I co-supervised in the laboratory (McCalister, 2015).

PerkinElmer LS55 Luminescence Spectrometer and Backlight Development software were used to determine fluorescence intensity in samples of cysts and pseudocysts. Mature cysts were prepared as previously described. Pseudocysts were prepared using NS with 10% methanol.

Samples were centrifuged, and the supernatant was discarded. Then, the pellet was resuspended in 1 ml buffer solution (20 mM Na-acetate pH 4.5 and Tris pH 6.0). Samples were incubated with 0.1 ml of 1 mM calcofluor white for 10 min. Samples were centrifuged and resuspended in 3 ml buffer. Measurements were taken using a quartz cuvette at 355 nm excitation and 442 nm emission with 2.5 nm slit widths.

2.9.7 Pseudocyst induction

Pseudocysts were induced using fresh amoebae culture and adding NEM with 10% methanol (Kliescikova et al., 2011b). The cultures were incubated at room temperature and monitored for the first three hours.

2.10 Bioinformatics

2.10.1 Basic local alignment search tool (BLAST)

A nucleotide BLAST was performed using the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with the obtained sequences (Altschul et al., 1990). The results were analysed to identify the species.

2.10.2 Alignments

Sequences were obtained by sequencing, GenBank and AmoebaDB (Aurrecoechea et al., 2011; Clark et al., 2016). Sequences were trimmed using MEGA 7. Alignments

were performed using MEGA 7 and the Muscle algorithm (Kumar et al., 2016). When needed, alignments were also created with Seaview 4.0 ClustalW to verify the results (Gouy et al., 2010).

2.10.3 Phylogeny tree

Phylogeny trees were constructed using a variety of software. First, they were performed using MEGA 7 constructing for maximum likelihood using the Kimura 2 parameter method bootstrapped between 100 and 500 replicates depending on the number sequences analysed (Kumar et al., 2016). These results were verified using Seaview 4.0 using the PhyML algorithm and the GTR model (Gouy et al., 2010). The non-parametric analysis was performed with 100 pseudo-replicates. When creating phylogenetic trees, species from different genera were used as outgroups to root the trees.

Bayesian inference was also used to create phylogenetic trees using MrBayes (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). The model used was the GTR substitution model with gamma-distributed and Markov chain Monte Carlo (MCMC) algorithms. The analysis started with 20000 generations, but new generations were added until reaching a standard deviation of split frequencies below 0.01. However, when performing analysis with a higher number of sequences and the standard deviation was unable to reach 0.01, the cut-off used was 0.05.

2.10.4 Sequence identity

The calculation of the sequence identity was performed using the software BioEdit (Hall, 1999). Results were compared to those from phylogeny trees. Sample comparisons with a similarity above 0.95 were highlighted.

2.10.5 Construction maps

Vector maps were created using SnapGene. For construction using the aLICator system, the In-Fusion function was used. For constructions using the Gateway system, the proper cloning function was used.

2.10.6 Hydropathy plots

Exploration of selected proteins to identify hydrophobic domains was performed using Membrane Protein Explorer (MPEx) software (Snider et al., 2009).

2.11 mRNAseq analysis

mRNAseq was performed to measure gene expression at four different time points during the encystment process: 0, 24, 48 and 72 hours after incubation with NEM. Strain 53, belonging to *A. castellanii* genotype T4, was used for the experiments. The cultures were grown in T25 flasks until confluent. Once confluent, the cultures were washed with PBS and NEM media was added to the culture. Cultures for 0 h treatment were immediately used for RNA extraction as previously described. The other cultures were incubated in NEM at room temperature until RNA extraction.

2.11.1 Sample quality control and quantification

Quality control was tested two different ways. First, agarose gels were run to observe the quality of the samples through comparison of ribosomal RNA following Edinburgh Genomics' guidelines. Second, QUBIT RNA BR (Broad-Range) Assay Kit (Thermo-Fisher Scientific) was used to quantify and test sample quality following manufacturer's instructions. Raw data quality control was performed using FASTQC (Andrews, n.d.).

2.11.2 Sequencing

Libraries were prepared for an automated TruSeq stranded mRNAseq from total RNA. The sequencing data generation was made with HiSeq-4000 75PE. These experiments were carried out by Edinburgh Genomics, The University of Edinburgh. Edinburgh Genomics is partly supported by core grants from NERC (R8/H10/56), MRC (MR/K001744/1) and BBSRC (BB/J004243/1).

Diagrams explaining the process of mRNAseq are shown in Figure 13 and Figure 14.

2.11.3 Mapping to reference genome

The reference genome (FASTA and GTF files) from *A. castellanii* was obtained from ENSEMBL Protists (Clarke et al., 2013; Kersey et al., 2017). The genome was indexed using STAR software. Later, the reads were aligned to the reference genome using STAR to obtain the required BAM files (Dobin et al., 2013). The alignments and the BAM files were visualised using SAMtools and IGV to verify the quality of the results (Li et al., 2009; Robinson et al., 2013).

2.11.4 Differential expression analysis

The differential expression analysis was performed using R studio and EdgeR (Robinson et al., 2009). A summary flowchart of the mRNAseq experiments can be found in Figure 13. The script used for the differential expression analysis can be found in Appendix 4.

Counts per gene were generated using featureCounts software with reverse stranded reads (Liao et al., 2014). The data was normalised using the trimmed median of M values or TMM (Robinson & Oshlack, 2010). Then, data was filtered applying counts per million (CPM) threshold of 0.1. The dispersion was estimated assuming that biological coefficient variation is constant. Later on, dispersion fitted to generalised linear models using the quasi-likelihood approach recommended by the EdgeR's authors.

The differential expression analysis was performed using Limma's "makeContrasts" and "topTags" functions (Ritchie et al., 2015). Differentially expressed genes were found using criteria of a fold change of 2 (Log2FC of 1) and an FDR threshold of 0.05. Genes of interest were selected.

Finally, differential gene expression set was performed using Generally Applicable Gene-set Enrichment (GAGE) obtaining the pathways for *Acanthamoeba* from the KEGG pathway gene sets (Luo et al., 2009).

The methods used are preferably used for paired groups, so most of the analysis was performed using the pair-wise comparison of the different treatments.

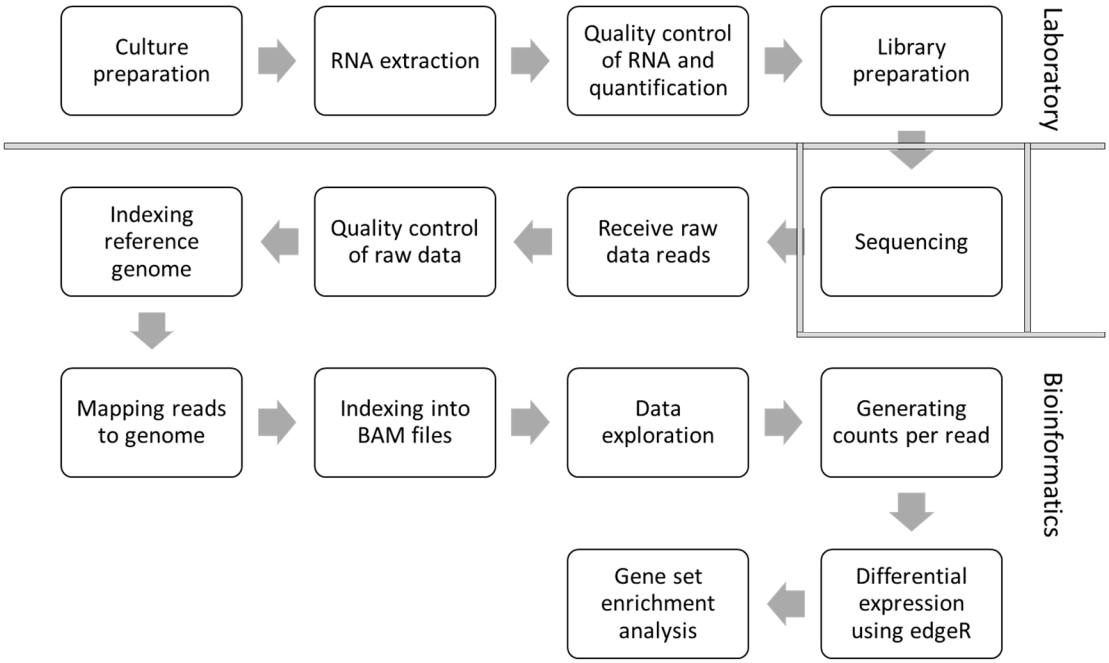


Figure 13. mRNAseq flowchart.

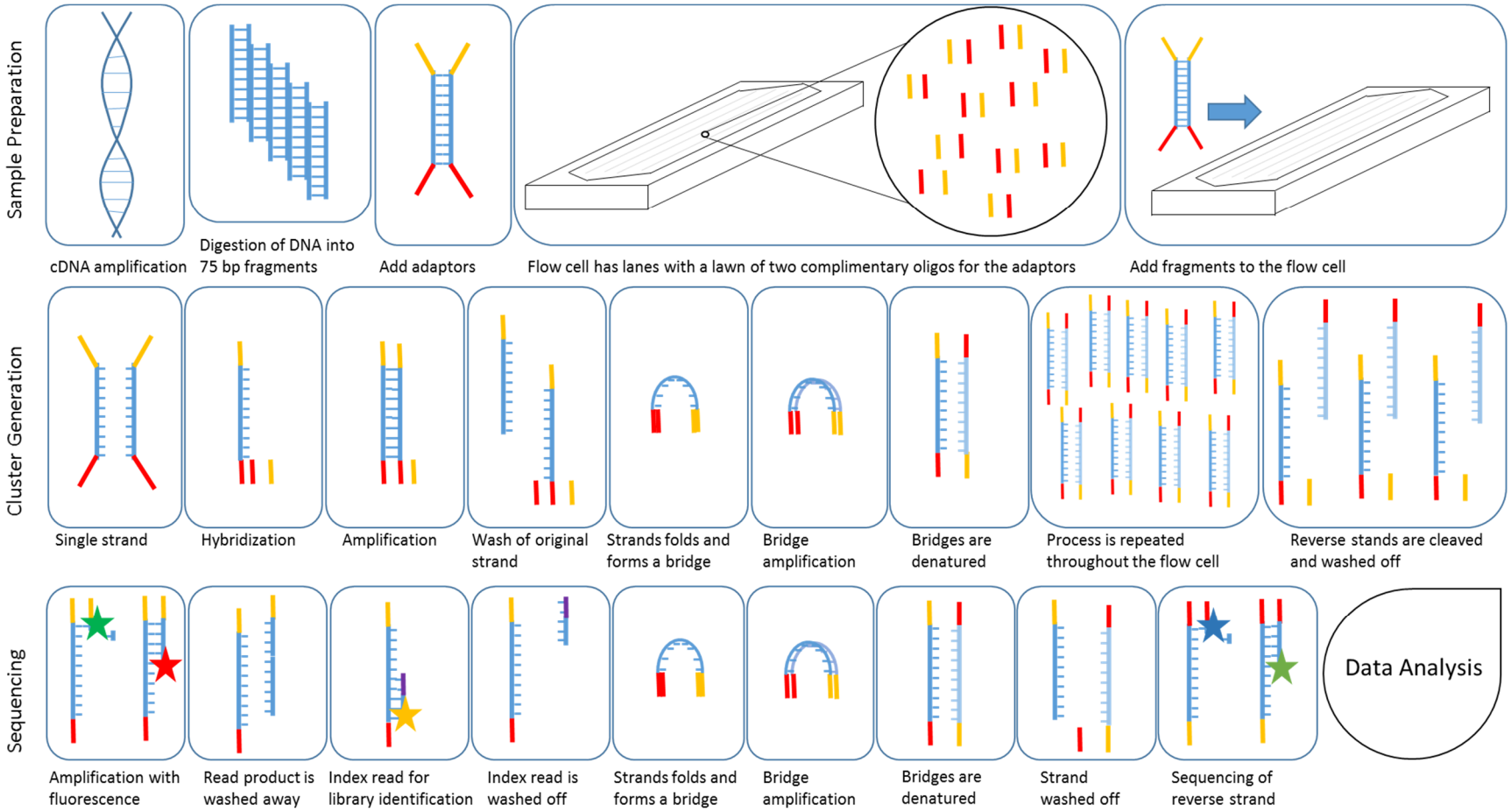


Figure 14. Diagram of mRNAseq

Chapter 3 *Acanthamoeba* biodiversity and phylogeny

3.1 Introduction

Biodiversity refers to the variety of living organisms and the different genetic compositions present throughout the world and history. The study of biodiversity is essential for several reasons, including the understanding of other areas of Biology such as Ecology, the formulation of public policy and the development of economic growth based on biotechnology (Bradley & Marciano-Cabral, 1996). The study of biodiversity is particularly important for amoebae since they are not monophyletic, therefore, in many cases, there is no phylogenetic relation. Amoebae present a vast range of characteristics since they are such a diverse group of organisms. The visual identification of amoebae can be very challenging because of their polymorphic bodies.

Figure 15 shows the different groups of amoebae in relation to each other and some of the most important groups of eukaryotes (Baldauf, 2008). It shows how amoebae are as diverse as most groups of Eukaryotes together, which can be appreciated for example by Amoebozoa being more closely related to animals than to other amoebae. All the amoebae can be classified into five major lineages Amoebozoa, Rhizaria (most are amoebae), Heterolobosea, Labyrinthula and Nucleariid amoebae (Brown & Silberman, 2013).

3.1.1 Amoebozoa

Amoebozoa consist of naked and testate amoebae and some other groups such as flagellates, Mycetozoa and Variosea. A simplified tree of the main groups forming Amoebozoa groups is presented in Figure 16. This section focuses on naked lobose amoebae, specifically *Acanthamoeba*. However, other representatives of Amoebozoa are studied in following chapters.

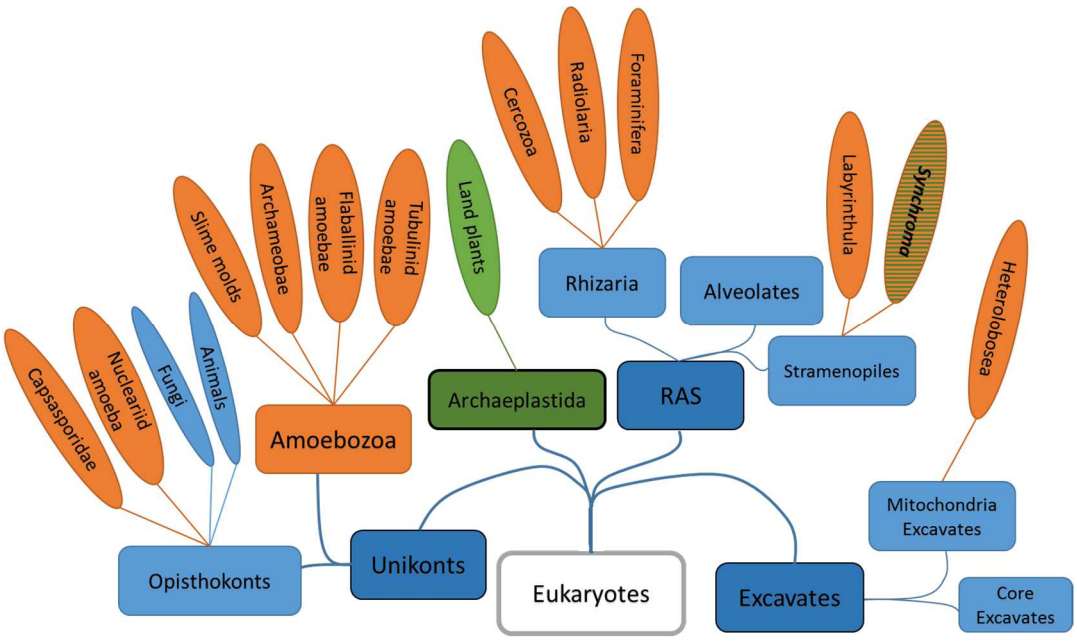


Figure 15. Summarized history of eukaryotes. The orange groups include amoeboid organisms. Modified from (Baldauf, 2008; Brown et al., 2012; Horn et al., 2007; Medina et al., 2003; Suga et al., 2013).

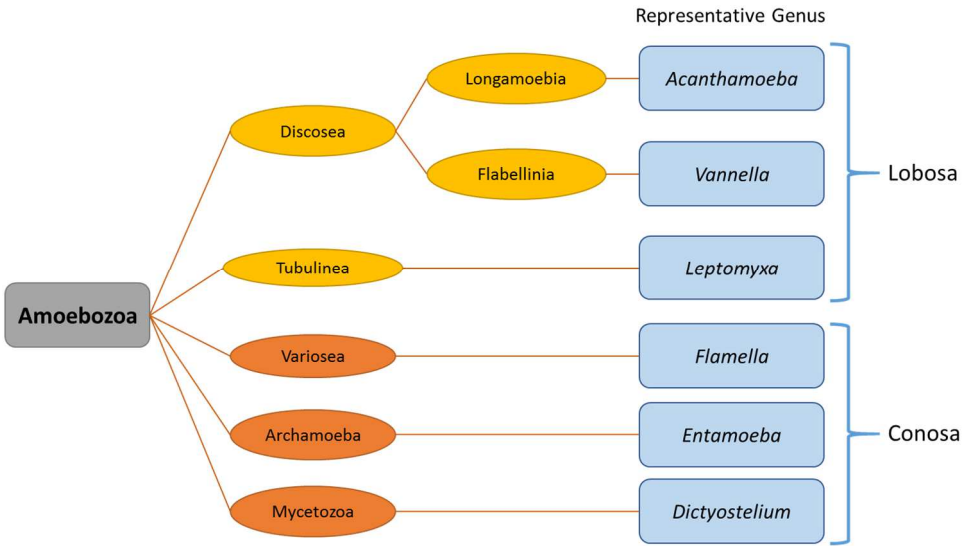


Figure 16. Relations between major groups of Amoebozoa. Modified from (Smirnov et al., 2011). It divides the lobose and conose amoebae. It also shows a representative genus from each major group.

3.1.2 Genus *Acanthamoeba*

Acanthamoeba is a cosmopolitan organism of importance to human health. The identification of different strains of *Acanthamoeba* helps with the understanding of the

biodiversity of amoebae, and it is important to the understanding of amoeba-bacteria relations (Walochnik et al., 1999).

Acanthamoeba is one of the most extensively studied amoebae. According to their morphology, they are divided into three morphogroups: I, II and III (Pussard & Pons, 1977). They have also been divided into 20 genotypes: T1 to T20 (Corsaro et al., 2015). The relationship between morphogroup, genotype and species is slightly confusing. In some cases, there are several species within a single genotype; in others, one species name is used for different genotypes. The classification of *Acanthamoeba* strains is based on the sequence of the small subunit 18S rDNA (SSU) gene (Corsaro et al., 2015; Corsaro & Venditti, 2011).

3.1.3 Chapter objectives

The study of *Acanthamoeba* diversity and phylogeny can help develop a more congruent, precise and comprehensive classification that can help better understand their biology. The identification of new strains of *Acanthamoeba* is particularly important since infections occur from environmental samples. Phenotypes grown axenically for long periods change, making the continuous isolation of environmental samples imperative.

3.2 Materials and Methods

A diagram of the materials and methods used is shown in Figure 17. A more detailed explanation of the protocols and experiments performed can be found in Chapter 2.

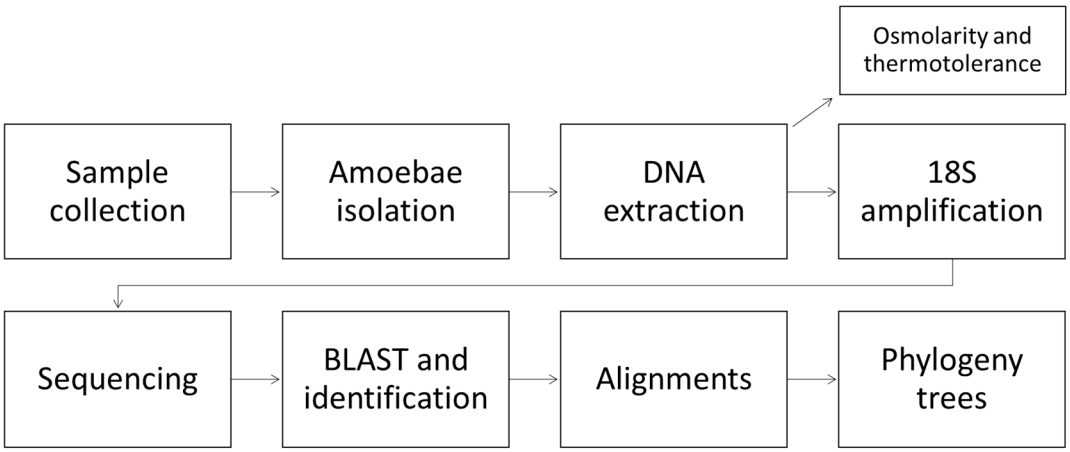


Figure 17. **Diagram of methods used in Chapter 3 for the isolation and identification of environmental** *Acanthamoeba* **strains.**

BLAST analysis was performed to compare the obtained sequences to the databases to perform the species identification. Afterwards, the sequences were aligned with other isolated samples and the sequences obtained from the database AmoebaDB (Aurrecoechea et al., 2011). Finally, the alignments were used to create the phylogeny trees. Since there are several methods to create these trees, three of the most common software were used to compare the results: MEGA, Seaview and MrBayes.

3.3 Results

Amoebae were isolated from different environments including fresh water, seawater, soil and organic material. Most of the samples were collected in Scotland. A summary of the *Acanthamoeba* strains used in this study including isolated and previously axenized strains is shown in Table 9.

Strain	Species	Isolation location	Specific	Substrate	Isolated by	Coordinates
NSS	*Acanthamoeba castellanii*	Isle of Skye	North coast	Marine	de Obeso	57°41'36.7"N 6°18'10.9"W
FF	*Acanthamoeba lenticulata*	Isle of Skye	Fairy Pools	Aquatic	de Obeso	57°15'01.1"N 6°15'29.9"W
BC	*Acanthamoeba lenticulata*	Baja California Sur	R. del Vizcaino	Lake bank	de Obeso	27°17'49.3"N 112°53'54.4"W
GS	*Acanthamoeba castellanii*	Edinburgh	George Square	Soil	Koutsagiannis	55°56'38.8"N 3°11'20.1"W
G5	*Acanthamoeba castellanii*	Edinburgh	George Square	Soil	Rodriguez	55°56'38.8"N 3°11'20.1"W
53	*Acanthamoeba castellanii*	Edinburgh	Silverburn	Soil	de Obeso	55°49'57.5"N 3°15'41.6"W
60	*Acanthamoeba royreba*	Previously isolated and axenized. No match to existing genotype				
61	*Acanthamoeba palestiniensis*	Previously isolated and axenized. ATCC 30870				
63	*Acanthamoeba polyphaga*	Previously isolated and axenized. ATCC 50372				
64	*A. castellanii* (Strain Galka)	Previously isolated and axenized. ATCC 50496				
65	*Acanthamoeba divionensis*	Previously isolated and axenized. ATCC 30137				

Table 9. *Acanthamoeba* strains used for this study.

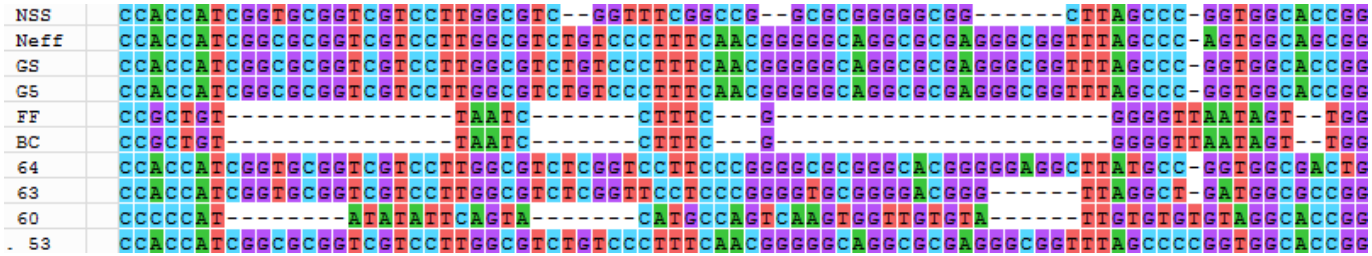


Figure 18. Small fragment of sequence differences between isolated *Acanthamoeba* species from the diagnostic ASA.S1 fragment. Strains FF and BC belong to the T5 genotype which are missing around ~40 bp of this fragment in comparison to strains belonging to the T4 genotypes

.

3.3.1 *Acanthamoeba* phylogeny and evolutionary trees

Ten different strains from *Acanthamoeba* were obtained and analysed. Of these, seven samples represented T4 genotype morphogroup II including the most commonly used laboratory strain known as Neff. The other three sample were members of morphogroup III with two belonging to genotype T5 and one to T2. The phylogenetic studies (Figure 19) corroborate the strain identification performed by BLAST analysis. The sequences used for the analysis can be found in Appendix 3.

Seq->	53	60	63	64	BC	FF	G5	GS	Neff	NSS
53	ID	0.842	0.938	0.935	0.79	0.811	0.981	0.962	0.957	0.941
60	0.842	ID	0.849	0.849	0.779	0.801	0.853	0.833	0.831	0.849
63	0.938	0.849	ID	0.975	0.798	0.821	0.946	0.926	0.922	0.946
64	0.935	0.849	0.975	ID	0.796	0.819	0.946	0.923	0.919	0.943
BC	0.79	0.779	0.798	0.796	ID	0.972	0.798	0.785	0.779	0.792
FF	0.811	0.801	0.821	0.819	0.972	ID	0.821	0.801	0.803	0.815
G5	0.981	0.853	0.946	0.946	0.798	0.821	ID	0.966	0.963	0.946
GS	0.962	0.833	0.926	0.923	0.785	0.801	0.966	ID	0.958	0.943
Neff	0.957	0.831	0.922	0.919	0.779	0.803	0.963	0.958	ID	0.941
NSS	0.941	0.849	0.946	0.943	0.792	0.815	0.946	0.943	0.941	ID

Table 10. Sequence identity matrix of *Acanthamoeba* isolates using full SSU sequences. Cells in red are those with a value above 0.95 meaning a high likelihood of belonging to the same species.

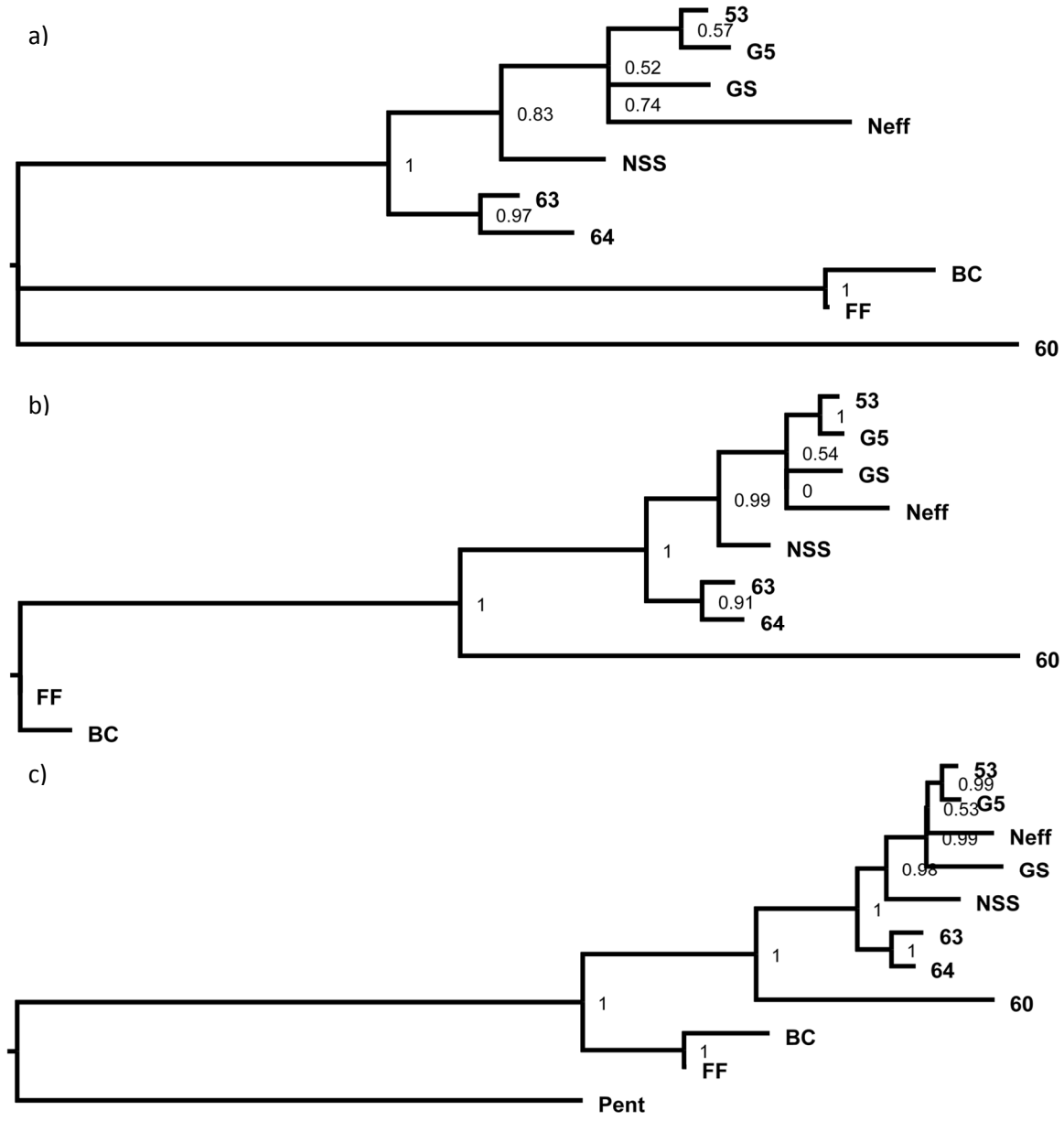


Figure 19. Phylogenetic tree for isolated strains of *Acanthamoeba* based on the full 18S rDNA gene. a) Phylogenetic tree using maximum likelihood method using MEGA 7. b) Phylogenetic tree using PhyML with the GTR model and 100 bootstraps using Seaview. c) Phylogenetic tree using Bayesian inference and 20000 simulated generations with Pent strain (*V. pentlandii*) as the outgroup used to root the tree.

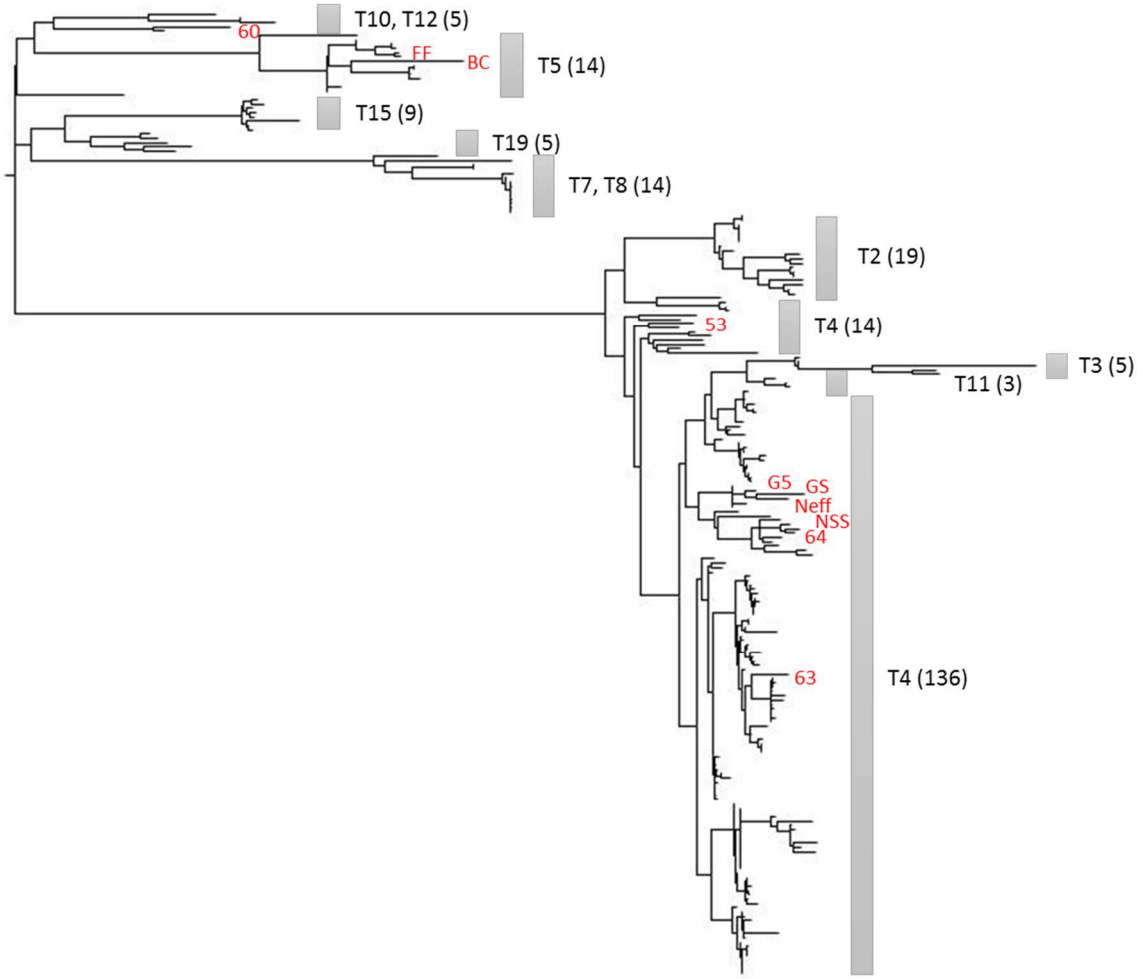


Figure 20. Phylogenetic tree of *Acanthamoeba spp.* based on the full 18S rDNA gene. The tree was created using 214 strains obtained from GenBank database and 10 sequences from isolated strains for this study. Sequences' names obtained from the database are missing for clarity, while isolated strains are marked. Clades are marked according to their genotype. The numbers in parenthesis refer to the amount of sequences included in each clade. The tree was created using PhylML with the software Seaview with model GTR and 100 bootstraps. Some of the submissions from GenBank had to be deleted since they did not align with the rest of the samples.

Figure 21. Phylogenetic tree of *Acanthamoeba spp.* based on the ASA.S1 fragment. The tree was created using 216 strains obtained from GenBank database and 10 sequences from isolated strains for this study. Sequences' names obtained from the database are missing for clarity, while isolated strains are marked. Clades are marked according to their genotype. The numbers in parenthesis refer to the amount of sequences included in each clade. The tree was created using PhylML with the software Seaview with model GTR and 100 bootstraps. Some of the submissions from GenBank had to be deleted since they did not align with the rest of the samples.

Phylogenetic trees created using the ASA.S1 and full 18S present clear clades. In both Figure 20 and Figure 21, the clades that can be clustered mainly belong to the same morphogroups. Genotypes T5, T10, T12 and T15 from morphogroup III are evolutionarily close. Meanwhile, the closest genotypes to T4 are T3, T11 and T19, which all belong to morphogroup II. Genotypes T7, T8 and T9 from morphogroup I are clustered in the same clade. However, there are not many records belonging to this group.

A quick search in GenBank for 18S genes from *Acanthamoeba* shows over 3000 hits. However, not all of them are complete or even belong to *Acanthamoeba*. The

information was filtered to include only records belonging to protists with an approximate size close to the full gene (between 1500 and 3000 were selected) which lowered the number of hits to 250. Of the resulting records, 216 sequences could be trimmed to isolate the ASA.S1 fragment, while 214 fragments had most of the SSU fragment. The trimming selection process was performed to have the most comparable trees between alignments.

The topology between phylogenetic trees varies slightly. However, topologies from trees obtained using the three methods are very similar.

3.3.2 Genotype identification of environmental isolated strains

The isolated species seem to group accordingly to the results of the higher phylogenetic analysis. A large group includes all strains belonging to the T4 genotype, and a smaller clade includes BC and FF, which belong to the taxon *A. lenticulata* and a T5 genotype. Strain 60 belongs to the T2 genotype separate from the others. T

According to BLAST search and analysis of the ASA.S1 fragment, strain 60 was classified in the T2 genotype. However, analysis of the full SSU and reconstruction of phylogeny by PhyML places strain 60 in a cluster including T10 and T12 genotypes and isolated from T2. The sequence identity matrix in Table 10 confirms this data.

3.3.3 Thermotolerance and osmotolerance of BC strain

Strain BC belongs to the genotype T5 also known as *A. lenticulata* and it was observed that it was capable of surviving at higher temperartures than the other strains. Pathogenic amoebae are normally capable of surviving at higher temperatures (37 ˚C) and salinity. The isolated strain was able to survive at 37˚C, up to 42˚C, for over 48 hours, which would be the first indication of possible pathogenicity, therefore further testing was required. However, the same strain was unable to survive in NS agar supplemented with mannitol, making it highly unlikely to be pathogenic. Initial experiments showed that BC strain grows faster for the first 24 h at 37 ˚C, and it levels off with room temperature growth afterwards. BC was even able to grow for some time at 42˚C, however at a slower rate than room temperature

and 37˚C. Results for growth at different temperatures over 48 hours can be seen in Figure 22.

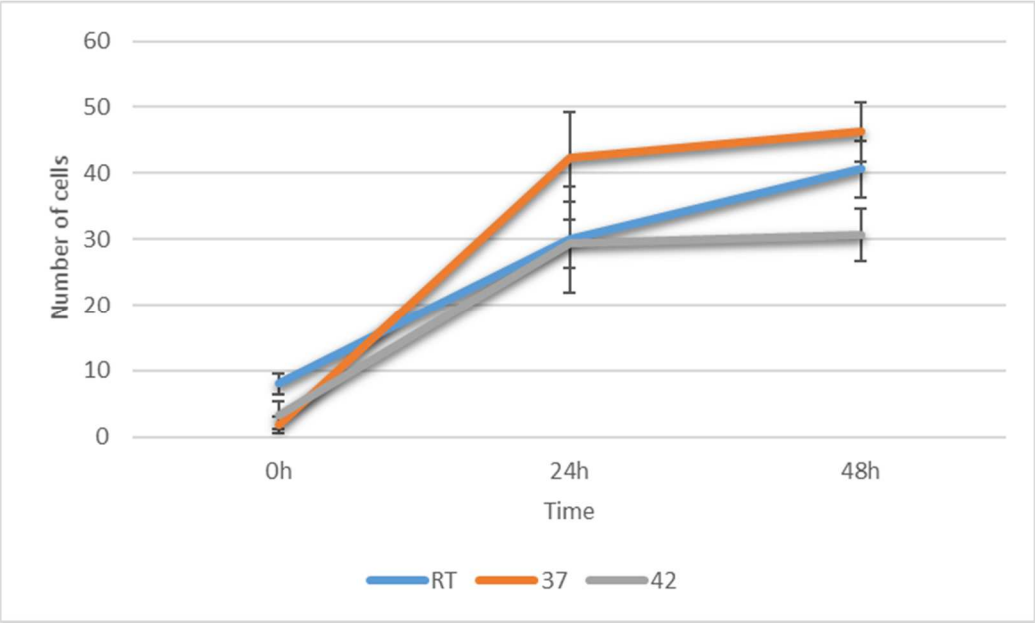


Figure 22. BC strain temperature growth comparison. Growth of BC strain after 24 and 48 hours at room temperature, 37˚C and 42˚C. The measurements shown are the average number of cells observed in six pictures from different cultures from each treatment. The error bars represent the standard error of the mean.

3.4 Discussion

Classification of *Acanthamoeba* used to rely on morphological characteristics and isoenzymes (Jonckheere, 1983). However, it is clear that morphological identification of amoebae is inadequate. For example, in the case of *Acanthamoeba*, it has been established that morphogroups II and III sometimes could not be distinguished morphologically (Visvesvara, 1991). Due to previous misidentification, there is still confusion and constant changes in *Acanthamoeba* systematics. Several studies have been published trying to make sense of all the conflicting information and looking to present a more congruent phylogeny for *Acanthamoeba* (Corsaro et al., 2015, 2017; Corsaro & Venditti, 2011; Jonckheere, 1983; Stothard et al., 1998; Visvesvara, 1991). However, it still is challenging to work through all the synonyms and inaccuracies present. For example, Liu showed that four species (*A. paradivionenesis, A. divionensis, A. mauritaniensis* and *A. rhysodes*) were synonyms and belonged to only one species (Liu et al., 2005). Additionally, some species names have routinely been

used to refer to different genotypes. One such case is *A. castellanii* that has members in genotypes T4 and T1. Even more, *A. castellanii* T1 has been reported as a member of morphogroups II and III while members of genotype T4 belong to morphogroup II (Stothard et al., 1998; Visvesvara, 1991).

The phylogenetic trees were congruent regardless of the approach, whether using maximum likelihood, PhyML or Bayesian inference. The clades remain similar throughout the different analysis. Some samples seemed to be in the wrong clade according to their species name, but this is likely due to an incorrect classification (Corsaro et al., 2015; Corsaro & Venditti, 2011; Stothard et al., 1998; Visvesvara, 1991). There are several instances of strains misidentified that belong to genotype T4 but were identified with species names belonging to other genotypes. Some such misidentified strains according to the phylogenetic trees in Figure 20 and Figure 21 belong to the T4 clade are identified as: *A. culbertsonii* (1X time), *A. hatchetii* (3x), *A. palestiniensis* (1X).

3.4.1 Comparison of the full 18S SSU rDNA or ASA.S1 fragment as means of species identification

It has been shown that the ASA.S1 fragment for *Acanthamoeba* identification is insufficient (Corsaro et al., 2015). However, the previous prevalence and lack of consistency in the databases make it a valuable tool for a quick and straightforward approach. However, the full gene still needs to be sequenced and compared for adequate identification (Corsaro et al., 2015). The GTSA.B1 (~1600 bp) should be the minimum requirement to make species identification, and the full SSU is still preferable as there can be variability throughout the whole gene (Schroeder 2001). However, ASA.S1 is the most widely available in databases (Corsaro & Venditti, 2011). This is corroborated by a quick search through GenBank which has almost 2000 records of 18S rDNA of the proper size for the ASA.S1 fragment. Only 10% of the records are close to the whole gene in size, or at least the GTSA.B1 required for adequate genotyping (Schroeder-Diedrich et al., 1998; Schroeder et al., 2001).

The similar results originating from the ASA.S1 and the full SSU phylogenetic trees show the value of the diagnostic fragment (Corsaro et al., 2010). However, the ASA.S1 value is limited besides diagnosis. It should stay as a diagnostic tool and

should not be incorporated as evidence for new taxa. The use of such fragment for genotyping and identification should be avoided since it can be inaccurate and the constant use of incomplete and unreliable data only increases confusion (Corsaro et al., 2015). This confusion is evident when creating phylogenetic trees, as there are prominent cases of species misidentification throughout.

One of the few advantages of using the ASA.S1 fragment is the number of sequences available. However, there is enough information to use the full SSU instead of the ASA.S1 fragment. There is a considerable difference in the amount of data for the ASA.S1 fragment compared to the full SSU (around 10 to 1 records in GenBank). However, the full SSU has been sequenced and reported for all genotypes, and the information has proven to be enough for phylogeny studies (Magnet et al., 2015). Even more, after alignment and selection of sequences of interest, the difference between the full gene and the diagnostic fragment was only of two samples out of more than 200. The difference of less than 1% is negligible.

ASA.S1 fragment does provide high genus specificity for *Acanthamoeba* and is obtainable from all genotypes. These characteristics make it ideal for diagnostic applications and the analysis of environmental samples when there are time constraints, and there are no axenic cultures (Schroeder 2001). However, obtaining axenized *Acanthamoeba* cultures can be time-consuming (some of the isolated cultures in this study took close to a year to be axenized), but the co-culture with *E. coli,* can be achieved relatively quickly (less than two weeks). The primers used for the amplification of the 18S rDNA only work with eukaryotes, therefore once in co-culture with *E. coli* it is relatively easy to amplify the full gene. Therefore, there are no sensible advantages to using the ASA.S1 diagnostic fragment outside a clinical setting (Schroeder et al., 2001).

3.4.2 Comparison of the isolated strains

Isolated T4 strains belong to the same species and are highly similar to Neff strain. The G5, GS and 53 strains differ slightly from Neff since the SSU from 18S rDNA is always variable (Nassonova et al., 2010).

A. lenticulata is tightly related to the T5 genotype (Schroeder-Diedrich et al., 1998). Both isolated *Acanthamoeba* T5 strains (BC and FF) came from water bodies. *A. lenticulata* has been described as being rare (Iovieno et al., 2010). However, two of the ten strains belonged to the T5 genotype which might indicate that it is common in the environment but could be uncommon for pathogenic strains. GenBank supports this information, where T5 / *A. lenticulata* are the second largest represented genotype clade after T4. A literature search on the relative frequency of occurrence in the environment and in human pathology also agrees with T5 being the second most common isolate (Maciver et al., 2013).

Using the full SSU gene sequence, strain 60 was identified by BLAST to belong to the T2 genotype. However, phylogenetic trees using the full SSU gene put it in a different clade. Nevertheless, using the ASA.S1 fragment exclusively, strain 60 is clustered with other T2 (Corsaro et al., 2015). These results are corroborated by the sequence identity matrix created. However, the identity matrix is dependent on the alignment and the identical residues present when the matrix is created (Hall, 1999).Therefore it might be ideal to perform the alignment using different methods that might reduce the influence of factors such as order sensitivity or information loss surrounding gap regions (Grasso & Lee, 2004). Results show that establishing evolutionary relations still has limitations and it is dependent on the software being used. Therefore, continuous work and development of phylogeny algorithms are required (Katoh & Standley, 2013).

Clarification of *Acanthamoeba* classification and phylogeny remains necessary. There is ample erroneous information in the databases, which provide unclear relations between species, genotypes and morphogroups. Just recently, two strains of genotype T19 have been reported with both morphology of group II and III (Corsaro et al., 2015; Magnet et al., 2015). Also, genotype T16 refers to two different amoebae while genotype T20 has been classified in morphogroups II or III also (Corsaro et al., 2015; Fuerst et al., 2015; Visvesvara et al., 2007).

Until 2015, the diagnostic fragment was still used continuously, so a more consistent classification will emerge as more information becomes available (Corsaro et al., 2015). As the criteria for *Acanthamoeba* identification is standardised the phylogeny of the genus will be more precise. As more scientists use the full SSU gene to

establish phylogeny and use the 5% cut-off for new species, the evolutionary history of *Acanthamoeba* will be easier to elucidate (Corsaro et al., 2015; Stothard et al., 1998).

Chapter 4 *Vannella* biodiversity and phylogeny

4.1 Introduction

The genus *Vannella* was created by Bovee to include flat, normally fan-shaped amoebae with a hyaline front edge during locomotion (Bovee, 1965). The main characteristic of *Vannella* organisms is the fan-shaped pseudopodia known as the lamella, which is the half-moon hyaline structure in front of the amoeba (Page, 1983, 1987). *Vannella* is an abundant genus found in a very diverse range of environments. It is particularly numerous in marine environments including anaerobic sediments, abyssal planes and hydrothermal chimneys (Sauvadet et al., 2010; Smirnov & Fenchel, 1996). However, *Vannella* organisms have also been isolated from plants, salt marshes, vegetables, fish, high salinity environments, biofilm, bat guano and even Antarctic environments (Amaral-Zettler et al., 2006; Anderson et al., 2003; Chavatte et al., 2016; Dyková et al., 2005; Hauer et al., 2001; Lasjerdi et al., 2011; Moran et al., 2007; Mulec et al., 1983).

Vannella is a member of the phylum Amoebozoa, subphylum Lobosa, class Discosea, subclass Flabellinia, order Glycostilda, family Vannellidae (Cavalier-Smith et al., 2016). Not many morphological differences exist between *Vannella* and some other Discosea members, making classification a challenge. However, molecular analyses show that morphology is not a phylogenetically relevant distinction. Sequencing of the first 18S rDNA from a vannellid organism (*V. anglica*) helped establish the dual origin of Discosea, where *Vannella* has a different lineage than *Acanthamoeba* and *Hartmannella*, which belong to Longamoebia (Sims et al., 1999). Figure 23 shows the evolutionary relations of *Vannella* relative to Eukaryotes and Amoebozoa.

Previously, members of the genus *Vannella* were often classified as *Platyamoeba* as they were two distinct genera. The primary classification factor between these two genera was the presence or lack of glycostyles, which are groupings of glycoproteins on the cell surface. However, this distinction is not phylogenetically relevant. Recently, organisms in these groups have been grouped into two new genera *Vannella* and *Ripella*. The main distinction between both genera is the size of the 18S rDNA (Smirnov et al., 2007).

As the number molecular studies of the genus increases, species previously considered to belong to other genera have been included in *Vannella*, including *Platyamoeba* and *Hyalodiscus simplex* (Sims et al., 2002; Smirnov et al., 2007). Besides the 18S rDNA, other genes have been tested to identify a more precise method of classification for *Vannella*. Vannellids' 18S fragment is highly conserved and inter-specific divergence is low. In some cases, inter-specific divergence can be lower than polymorphisms found in clones from the same strain (Smirnov et al., 2007). Therefore, gene sequences with higher variability are required to assess phylogeny. Sequencing from the Internal Transcribed Spacer (ITS) did not provide congruent results relative to those expected from the 18S fragment (Dyková et al., 2005). Later, the cytochrome oxidase subunit 1 (COI) gene was identified and proposed as the best way to define new *Vannella* species and their evolutionary relations (Nassonova et al., 2010).

In 2000, *V. persistens* was discovered and identified as the first member of the genus capable of forming cysts (Smirnov & Brown, 2000). Only a few other members of the genus are capable of producing cysts including *V. schaefferi*, *V. placida*, *V. danica*, *V. contorta* and another isolate from Iran (Lasjerdi et al., 2011; Moran et al., 2007; Page, 1968; Smirnov et al., 2002).

4.1.1 Chapter objectives

During the isolation of amoebae from environmental samples, it is common to encounter vannellids. They are particularly common in Scotland (Maciver et al., 2017; Smirnov & Brown, 2000). The study of *Vannella* organisms is important as they play an essential role in nutrient availability in the environment. As many other FLA, *Vannella* are capable of carrying potential pathogenic intracellular parasites such as *Legionella*, making it more important to study species capable of encystment (Corsaro et al., 2013; Hoffmann et al., 1998; Kuroki et al., 1998; Michel et al., 2000). Understanding the biology of vannellids can help understand similar organisms.

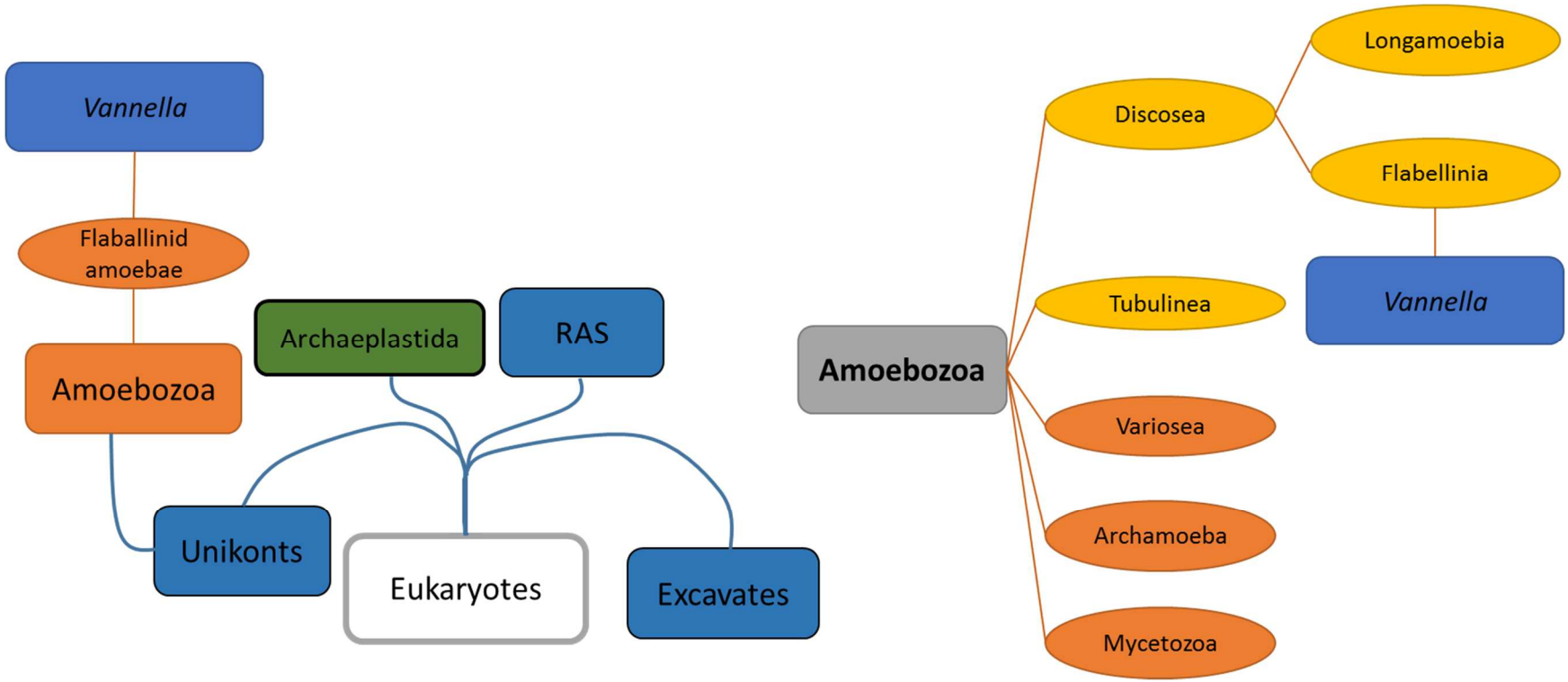


Figure 23. *Vannella* phylogeny. On the left, it shows its relation to other Eukaryotes. On the right, it shows its relation to other Amoebozoa organisms.

Strain	Species	Isolation location	Specific	Substrate	Isolated by	Coordinates
Pent	***Vannella pentlandii***	Scotland	Pentland hills	Soil	Maciver	55°49'57.5"N 3°15'41.6"W
LN	*Vannella sp.*	Highlands	Loch Ness	Loch bank	de Obeso	57°08'47.4"N 4°40'43.9"W
KC	*Vannella sp.*	Highlands	Kincraig	Soil	de Obeso	57°07'36.1"N 3°55'34.1"W
KR	*Vannella sp.*	Isle of Skye	Kilt Rock	Marine	de Obeso	57°36'38.5"N 6°10'19.0"W
Cow1.2	*Vannella sp.*	Midlothian	Cow	Cow faeces	Maciver	55°49'01.5''N 3°15'07.3' W
Cow2.1	*Vannella sp.*	Midlothian	Cow	Cow faeces	Maciver	55°49'01.5''N 3°15'07.3''W
Sh	*Vannella sp.*	Midlothian	Sheep	Sheep faeces	Maciver	55°49'01.5''N 3°15'07.3'' W
Arn	*Vannella sp.*	Scotland	Loch Hourn	Sycamore bark	Maciver	57°07'55.3''N 5°33'34.4'' W

Table 11. *Vannella* organisms isolated and used for this study. The species in bold letters is a newly reported species.

4.2 Materials and Methods

A diagram of the materials and methods used is shown in Figure 24. A more detailed explanation of the protocols and experiments performed can be found in Chapter 2.

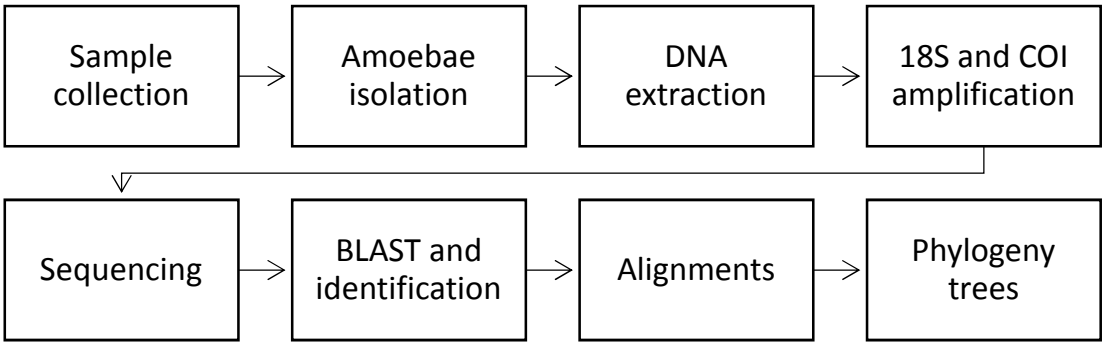


Figure 24. Diagram of methods used in Chapter 4 for the isolation and identification of environmental *Vannella* strains.

4.3 Results

Eight *Vannella* strains were isolated from different samples collected throughout Scotland. Of the eight samples, Sutherland Maciver isolated five. The strains came from several diverse environments including solid, freshwater, marine sediment and animal faeces. Table 11 shows the collection information for the *Vannella* strains used for this study. These strains' 18S rDNA gene was sequenced. In most cases, the COI gene was sequenced too. The sequencing results were aligned to records obtained from GenBank to establish phylogenetic relationships between all the available species and strains. A new cyst-forming amoeba was discovered and named *Vannella pentlandii* (Maciver et al., 2017). Examples of two of the isolated organisms can be seen in Figure 25.

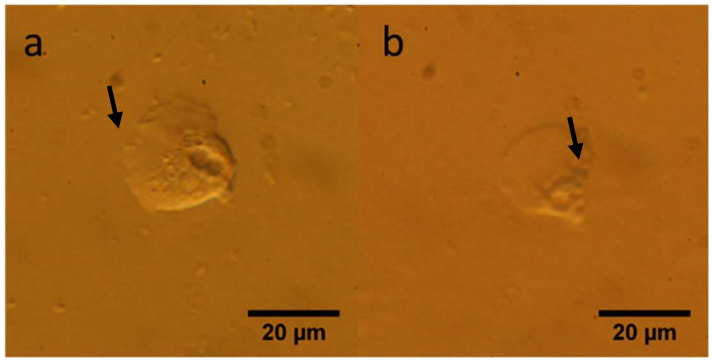


Figure 25. Images of *Vannella* species isolated from the Scottish Highlands. a) *Vannella sp.* isolated from Kincraig, Scotland. b) *Vannella sp.* isolated from Loch Ness, Scotland. Arrows point toward the lamella of the amoebae.

Measurements for three strains were taken and compared to some of the most common *Vannella* strains. The results can be found in Table 12.

Strain	Length (µm)		Width (µm)		Reference
	Min	Max	Min	Max	
V. danica	25	50	25	50	(Smirnov et al., 2002)
V. epipetala	20.2	28.6	23.4	37.1	(Amaral-Zettler et al., 2006; Todd et al.,
V. persistens	23	35	23	40	(Smirnov & Brown, 2000)
V. placida	15	35	9	66	(Page, 1968; Sims et al., 2002)
V. contorta	12	29	12	23	(Moran et al., 2007)
V. pseudovannella	8.5	25.5	6.8	25.5	(Hauer et al., 2001)
V. ebro	25	40	35	60	(Smirnov, 2001)
V. pentlandii	14	30	11	20	(Maciver et al., 2017)
LN	16	36	14.5	28	This study
Kc	18.5	40	18	33	This study
Arn	15.5	49	13.5	43	This study

Table 12. Size comparison of different *Vannella* strains. The table was modified from (Maciver et al., 2017).

4.3.1 **Phylogeny**

Phylogeny analyses were performed two different ways. First, including isolated strains and selected sequences from each of the following species: *V. simplex, V. persistens, V. danica, V. calycinucleolus, V. bursella* and *V. arabica*. The organisms were selected when information for 18S rDNA and COI gene was available to use as samples. Second, using data obtained from isolated strains and all the sequences

that could be aligned appropriately obtained from GenBank belonging to freshwater *Vannella*. Sequences obtained from isolated strains can be found in Appendix 3.

GenBank had 191 sequences related to the 18S rDNA fragment from different *Vannella* organisms. Eighty-two sequences were selected for analysis as they had most of the gene. Of the selected sequences, 46 were from strains isolated from freshwater that could be aligned and analysed with the sequences obtained after isolation. The selected strains included *V. pentlandii*, which was sequenced and uploaded into GenBank with an accession number of KY344796. Phylogeny trees for all the sequences were created using maximum likelihood approximation, and Bayesian approximation. *Paravannella minima* was included in the analysis and, as expected, resulted in a completely different isolated clade. *Acanthamoeba* NSS strain was used to root the phylogeny trees as an outgroup. Phylogeny trees and sequence identity matrix using sample sequences can be found in Figure 26 and Table 13 respectively.

GenBank had 71 records from *Vannella* of the COI gene that could be appropriately aligned with the ones obtained from sequencing. The selected strains included *V. pentlandii,* whose COI gene was sequenced and uploaded into GenBank with an accession number KY344797. A sequence identity matrix and phylogeny trees of the isolated COI sequences with sample sequences for some other *Vannella* species can be found in Table 14 and Figure 27 respectively.

The analysis of the 18s rDNA and COI gene shows that several of the isolated strains belong to the same clade. According to the phylogeny trees from both genes and the sequence identity matrix *V. arabica*, *V. bursera* and *V. calycinucleolus* form a single clade denominated here ABC. A similar situation happens with the clade that is formed between *V. simplex* and *V. persistens*, where they are closely related. There is another clade including four different strains isolated (Arn, Sh, Cow2.1 and Cow1.2).

Bootstrap values confirm a clade including the Sheep, Cow1.2, Cow 2.1 and Arn strains accompanied by previously reported *Vannella* species not yet named or identified. This clade was denominated ACS (Arn-Cow-Sheep). ACS clade is closest related clade to the marine amoebae group that includes *V. contorta, V.*

septentrionalis, V. devonica, V. australis, V. ebro, V. calcynucleolus, V. arabica and *V. bursella* (Mayes et al., 1997; Page, 1974, 1980).

Two other newly isolated strains, LN and KC, were also closely related. They belong to a clade including *V. planctonica*, *V. croatica*, *V. lata* and *V. miroides*. All these species have been isolated from freshwater samples.

V. pentlandii and *Vannella sp.* strain LN are capable of forming simple cysts.

Seq->	*Vsimplex*	Cow21	*Vpersistens*	*Vpentlandii*	*Vdanica*	*Vcalycinucleolu*	*Vbursella*	*Varabica*	NSS	LN	Sheep
Vsimplex	ID	0.792	0.971	0.828	0.92	0.886	0.886	0.882	0.603	0.779	0.819
Cow21	0.792	ID	0.79	0.808	0.795	0.778	0.779	0.777	0.591	0.795	0.924
Vpersistens	0.971	0.79	ID	0.825	0.924	0.883	0.886	0.882	0.601	0.778	0.815
Vpentlandii	0.828	0.808	0.825	ID	0.81	0.798	0.798	0.796	0.607	0.817	0.814
Vdanica	0.92	0.795	0.924	0.81	ID	0.866	0.869	0.864	0.606	0.776	0.82
Vcalycinucleolus	0.886	0.778	0.883	0.798	0.866	ID	0.987	0.987	0.603	0.791	0.81
Vbursella	0.886	0.779	0.886	0.798	0.869	0.987	ID	0.994	0.605	0.79	0.811
Varabica	0.882	0.777	0.882	0.796	0.864	0.987	0.994	ID	0.605	0.789	0.809
NSS	0.603	0.591	0.601	0.607	0.606	0.603	0.605	0.605	ID	0.608	0.604
LN	0.779	0.795	0.778	0.817	0.776	0.791	0.79	0.789	0.608	ID	0.799
Sheep	0.819	0.924	0.815	0.814	0.82	0.81	0.811	0.809	0.604	0.799	ID

Table 13. Sequence identity matrix for the 18S sequence. Obtained from selected and isolated *Vannella* strains with BioEdit. Strains that have a higher than 0.95 similarity index are highlighted in red.

Seq->	NSS	Cow1.2	*Vpentlandii*	*Varabica*	*Vbursella*	*Vcalcynucleo*	*Vdanica*	*Vpersistens*	*Vsimplex*	LN
NSS	ID	0.721	0.737	0.731	0.731	0.737	0.731	0.733	0.737	0.757
Cow1.2	0.721	ID	0.686	0.763	0.761	0.761	0.807	0.821	0.817	0.765
Vpentlandii	0.737	0.686	ID	0.688	0.69	0.711	0.729	0.721	0.735	0.719
Varabica	0.731	0.763	0.688	ID	0.989	0.921	0.803	0.799	0.803	0.769
Vbursella	0.731	0.761	0.69	0.989	ID	0.925	0.801	0.795	0.799	0.767
Vcalcynucleolus	0.737	0.761	0.711	0.921	0.925	ID	0.799	0.797	0.797	0.775
Vdanica	0.731	0.807	0.729	0.803	0.801	0.799	ID	0.874	0.886	0.777
Vpersistens	0.733	0.821	0.721	0.799	0.795	0.797	0.874	ID	0.927	0.775
Vsimplex	0.737	0.817	0.735	0.803	0.799	0.797	0.886	0.927	ID	0.767
LN	0.757	0.765	0.719	0.769	0.767	0.775	0.777	0.775	0.767	ID

Table 14. Sequence identity matrix for the COI sequence. Obtained from selected and isolated *Vannella* strains with BioEdit. Strains that have a higher than 0.95 similarity index are highlighted in red.

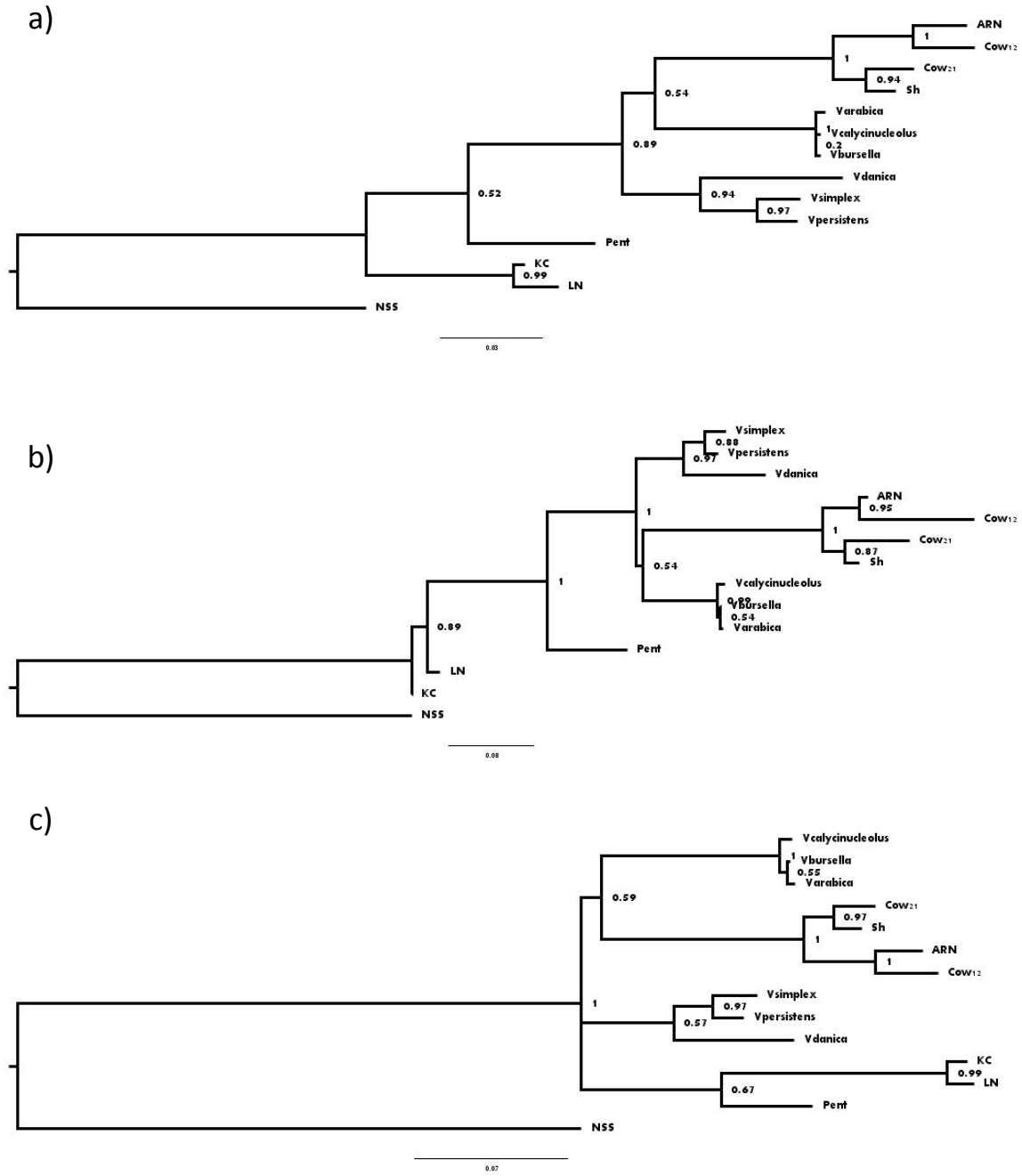


Figure 26. *Vannella* phylogeny based on 18S rDNA. *Acanthamoeba* NSS strain was used to root tree as the outgroup. a) Phylogeny tree using maximum likelihood (Kimura 2-parameter model) and MEGA 7. b) Phylogeny tree using PhyML (GTR model) and Seaview. c) Phylogeny tree using Bayesian approximation and MrBayes with 300,000 generations.

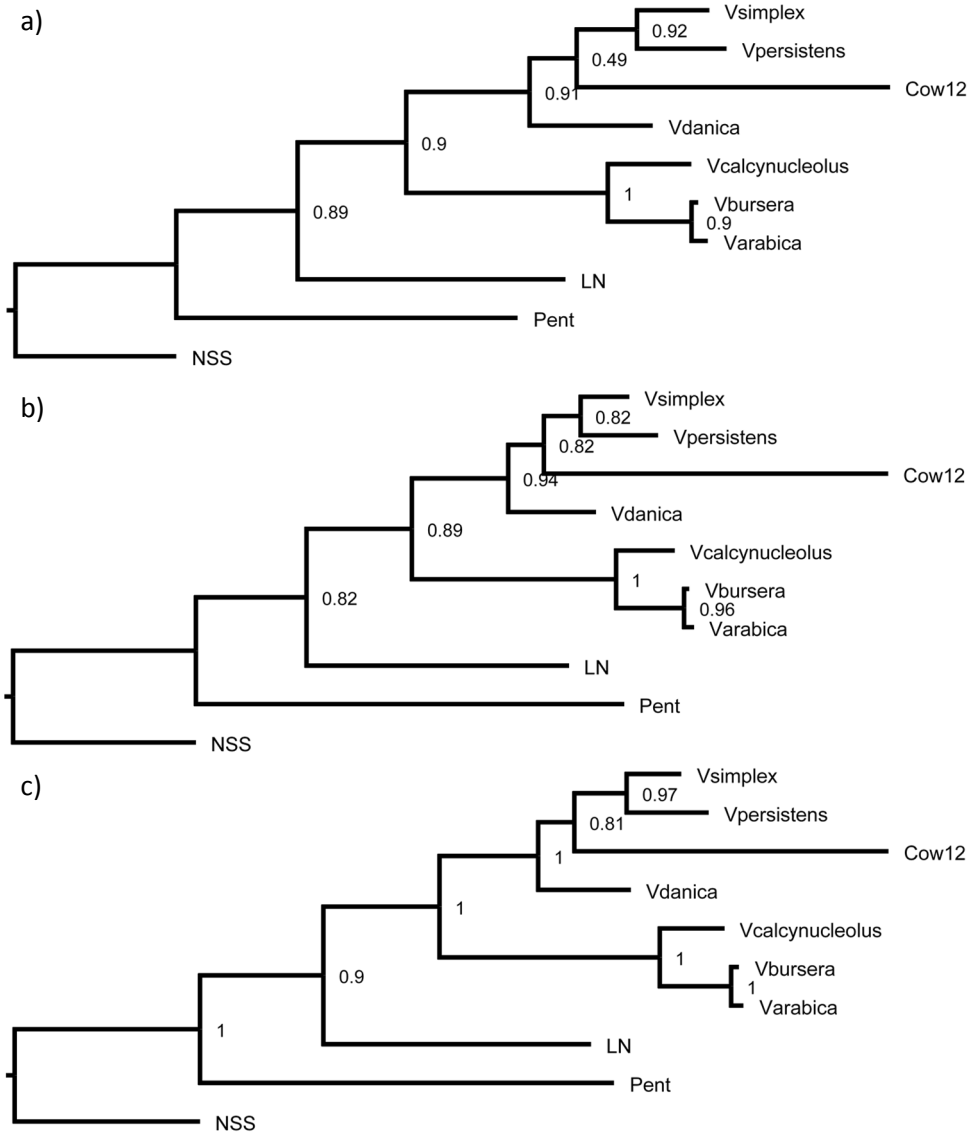


Figure 27. Phylogenetic trees of *Vannella spp.* using the COI gene. Sequences were obtained from GenBank database and isolated samples. NSS *Acanthamoeba* sequence was used as the outgroup to root the trees. a) Phylogenetic tree using maximum likelihood method created using MEGA 7 with the Kimura 2-parameter model and 100 bootstraps. b) Phylogenetic tree using PhyML with the GTR model and 100 bootstraps created using Seaview. c) Phylogenetic tree using Bayesian inference with MrBayes until the standard deviation of split frequencies was below 0.01.

4.4 Discussion

Vannella phylogeny has been extensively studied. *Vannella* is a diverse genus where phylogenetic analysis has shown a clear separation between marine species and freshwater/soil species (Sims et al., 2002; Smirnov et al., 2007).

The concept of species regarding amoebae is ill-defined, more so in *Vannella* due to the microheterogeneity found in the 18S rDNA fragment. Therefore, the use of other gene sequences as bases for classification such as the COI gene is important (Nassonova et al., 2010). The phylogeny results obtained from analysis of the 18S rDNA and COI genes are similar (Maciver et al., 2017). In actuality, it is best practice to sequence both fragments. Although the COI gene could provide better phylogeny results, there is still limited information regarding the *Vannella's* COI gene . When databases provide enough information for the COI gene to become a real option for analysis, it could become the first criterion for *Vannella* identification.

Due to the lack of information regarding *Vannella* COI gene and the difficulties presented by 18S rDNA, different approaches should be used to identify species. Sequence identity matrixes can serve as a guideline in conjunction with phylogeny trees. However, results from the 18S rDNA confirm that intra-species variability can affect phylogeny studies (Smirnov et al., 2007). Only organisms from *V. epipetala* frequently showed identity recognition values over 0.95. Nevertheless, Nassonova et al. suggested the use of the COI gene to clarify lobose amoebae phylogeny. Results from the sequence identity matrix obtained from the COI gene did manage to separate several species in which using the 18S sequence was not enough (Nassonova et al., 2010). Unlike 18S rDNA sequences, phylogeny based on COI sequence was able to divide clade ABC. It distinguished *V. calycinucleolus* as a separate species, as well as differentiating between *V. simplex* and *V. persistens*. Clade ABC is particularly contentious since their morphological characteristics are not congruent with the molecular results. *V. bursella* and *V. arabica* are considered different species based on their morphological differences. However, the phylogeny does not support these claims. These three species have very distinctive morphological differences where *V. calycinucleolus* is larger, while *V. bursella* has very distinct locomotion. The COI sequence helps elucidate a little bit more as it separates them slightly.

The use of a Molecular operational taxonomic unit (MOTU) has been suggested to establish the divergence between species. MOTU can help when some organisms of the same species (*V. simplex*) tend to be more dissimilar between them than different species like *V. arabica* and *V. bursella* are between them (Floyd et al., 2002).

The ACS clade is formed by three isolated strains and four strains from GenBank not identified (AY929909, AY929910, AY929911 and JQ271731). All these strains, except Arn, were isolated from animals or animal residues (Dyková et al., 2005). However, the animals are very varied as they are cow, sheep, freshwater fish and sea urchin. The marine strain (JQ271731) was from inshore location, which could explain its grouping with freshwater/soil strains (Maciver et al., 2017).

Besides the seven strains within, the ACS clade is most closely related to *V. simplex* and *V. persistens*. Coincidentally, *V. persistens* was first isolated in Scotland too (Smirnov & Brown, 2000). However, there does not appear to be a relationship between cyst forming amoebae. *V. persistens* is closely related to *V. danica*, and *V. pentlandii* to *V. placida,* but cysts do not seem to have phylogenetic relevance in *Vannella*. Therefore, it is probable that the ability to produce cysts is an ancestral property of the Amoebozoa since several organisms present it (*Acanthamoeba* for example) and the differentiation shows similar characteristics. It is possible that some species have lost the capacity to form cysts altogether, while some others might have lost it, and reacquired it with time (Maciver et al., 2017).

Most BLAST results from the isolated strains showed results related to *Platyamoeba* strains. However, this genus was merged into *Vannella* when it was proven that the presence of glycostyles had no molecular relevance (Smirnov et al., 2007). Nevertheless, the genus *Platyamoeba* is still widely used (Ramirez et al., 2010, 2014; Todd et al., 2015).

Learning more about *Vannella* biology might help elucidate different characteristics of amoebae. It could help understand the amoebae-bacteria relation that plays an essential role in the environment and in many human diseases. Since *Vannella* feeds on bacteria and can harbour some of them, a more in-depth understanding might provide an alternative to deal with some of these pathogens (Schulz et al., 2015).

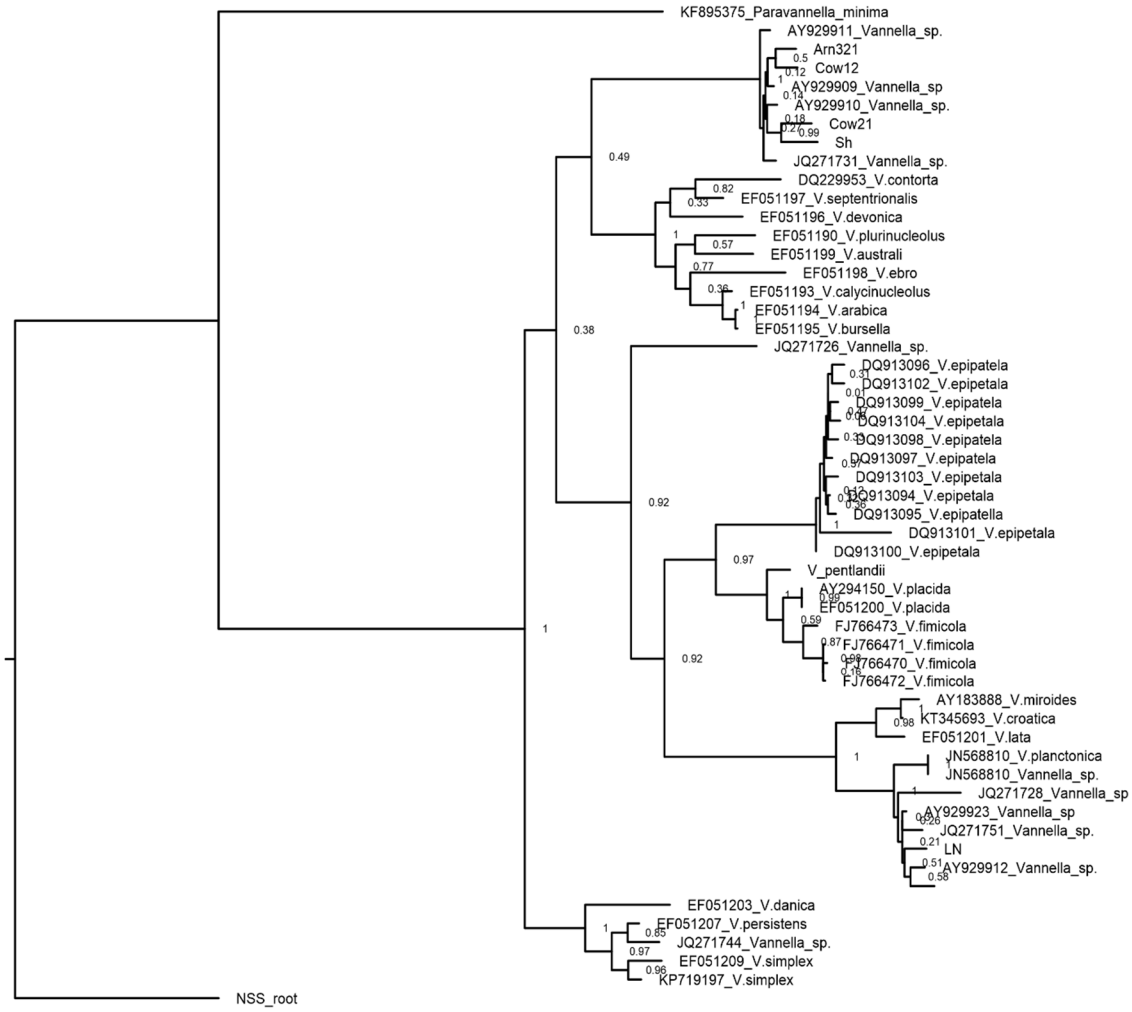


Figure 28. Phylogeny trees using maximum likelihood method using MEGA 7 of *Vannella spp.* using 18S rDNA. The tree was created using a Kimura 2-parameter model and 100 bootstraps. The samples selected were the from freshwater organisms that could be aligned from sequences obtained from GenBank database and isolated samples. NSS *Acanthamoeba* sequence was used as the outgroup to root the trees.

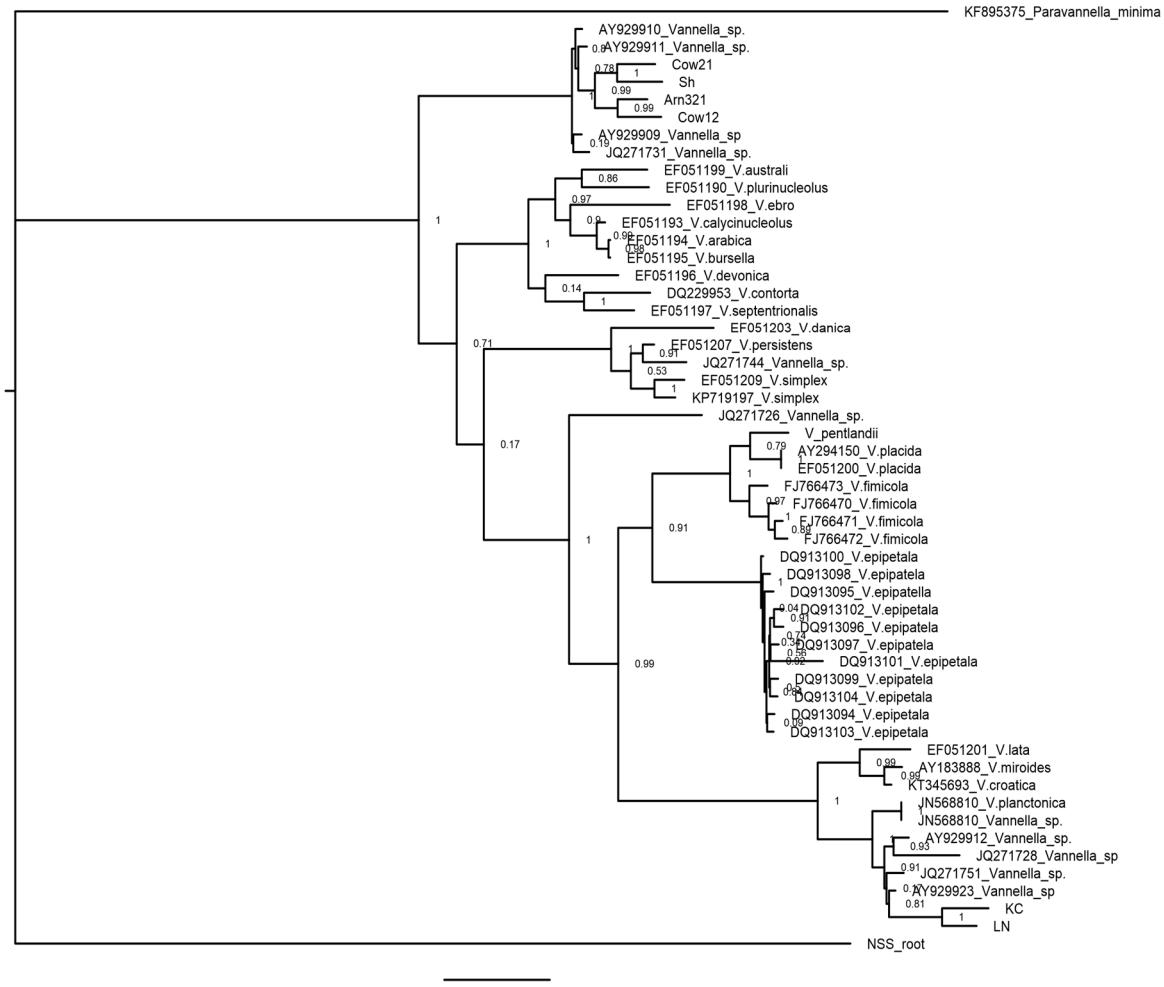


Figure 29. Phylogeny tree made using PhyML of *Vannella spp.* based on the 18s rDNA gene using SeaView software. The tree was created using a GTR model and 100 bootstraps. The samples selected were from freshwater organisms that could be aligned from sequences obtained from GenBank database and isolated samples. NSS *Acanthamoeba* sequence was used as the outgroup to root the trees.

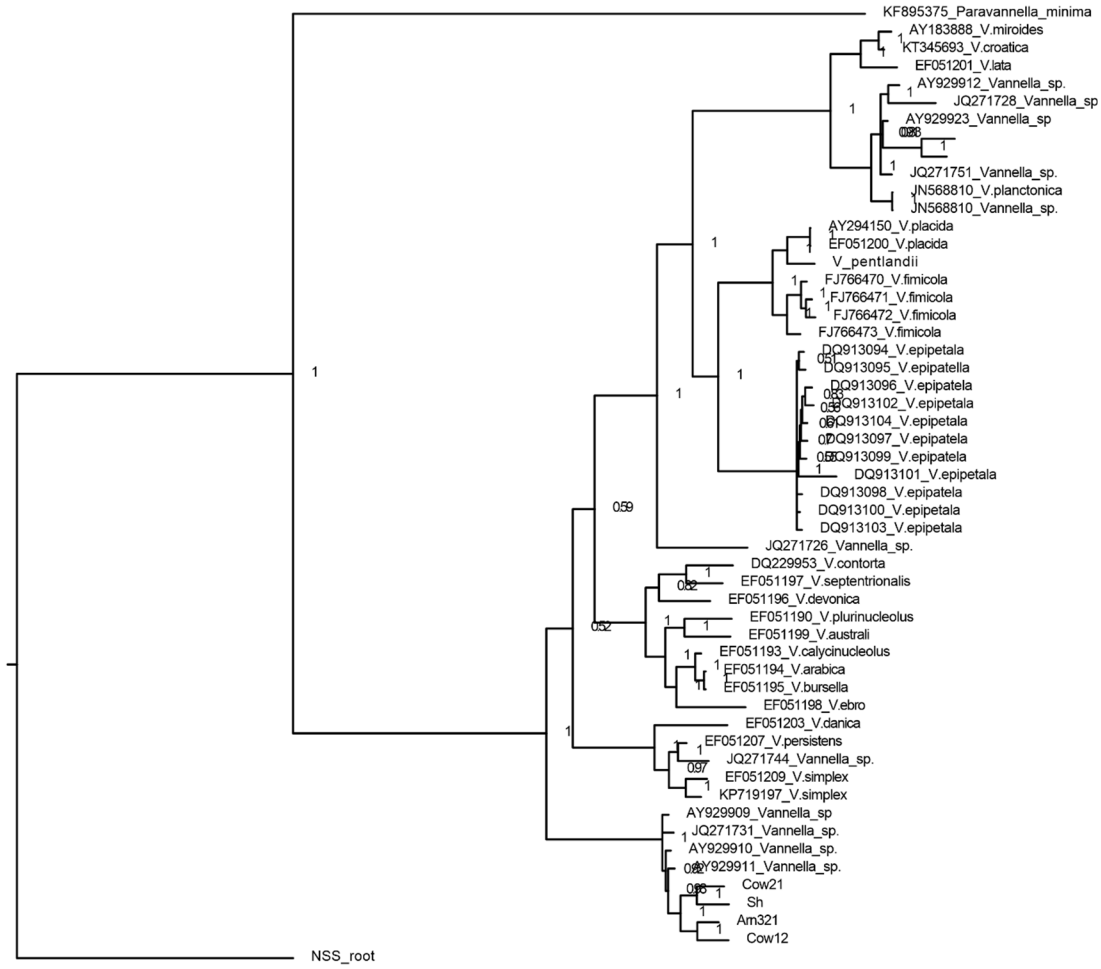


Figure 30. Phylogeny tree using Bayesian approximation with MrBayes of *Vannella spp.* based on the 18S rDNA. 750,000 generations were used to obtain a standard deviation of split frequencies under 0.01. The samples selected were from freshwater organisms that could be aligned from sequences obtained from GenBank database and isolated samples. NSS *Acanthamoeba* sequence was used as the outgroup to root the trees.

Chapter 5 Other isolated amoebae

5.1 Introduction

Understanding the biodiversity of FLA is essential since they play an essential role in the environment. FLA are a polyphyletic group, with different characteristics. The isolation and culture of different amoebae offer the possibility to find new organisms. For this study, two new amoebae were isolated and identified: a leptomyxid and a heterolobosean. The two new amoebae discovered and described for this study are *Leptomyxa valladaresi* and *Allovahlkampfia minuta* (de Obeso Fernandez del Valle et al., 2017; de Obeso Fernandez del Valle & Maciver, 2017). A simplified history of eukaryotes and the evolutionary location of both amoebae can be found in Figure 31. The results and discussion can be found in the articles in Appendix 1.

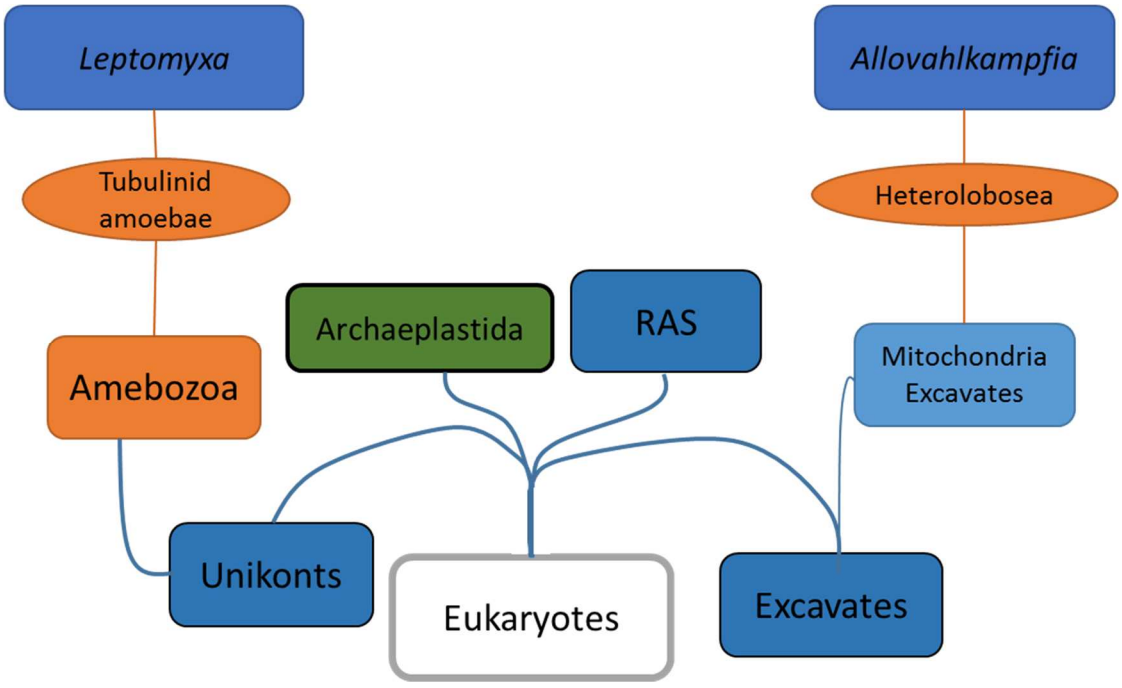


Figure 31. Diagram indicating the evolutionary position in regards to other Eukaryotes of the genera *Leptomyxa* and *Allovahlkampfia*.

5.1.1 Family Leptomyxidae

Amoebozoid organisms of the Leptomixidae family are medium to very large amoebae some of which can be up to 3 mm long making them some of the longest amoebae (Singh, 1948). Goodey was the first to describe Leptomyxidae amoebae (Goodey, 1915). Leptomyxidae amoebae are found in a variety of environments such as freshwater (Smirnov et al., 2009), marine environments (Page, 1983), temperate soil (Brown S & Smirnov AV, 2004), permafrost soil (Shmakova et al., 2014) and desert soil (Bamforth, 2008). All leptomyxids have a simple life cycle that includes a trophozoite and a cyst. Leptomyxids present different morphologies even within the same species. They can be fan-shaped, flattened, have limax or ramose form, and even the same organism can change its locomotive morphology. An important characteristic for all Leptomyxids is the capacity to develop an adhesive uroid (Smirnov et al., 2017). As with many other amoebae groups, and as a result of their varied morphologies, Leptomyxidae has been a group in constant flux, and its classification is a challenge (Smirnov et al., 2009, 2008). After a recent reorganisation of the family, Leptomyxidae has three recognised genera: *Flabelulla*, *Rhizamoeba* and *Leptomyxa* (Smirnov et al., 2017). Leptomyxidae belongs to the class Tubulinea.

Presently, it is clear that members of the genus *Leptomyxa* and related groups are not pathogenic. *Balamuthia madrillaris*, one of the primary causatives of amoebic encephalitis, was initially classified as a leptomyxid due to its superficial resemblance to the group (Visvesvara et al., 1990). However, subsequent studies showed primary differences and showed that *B. mandrillaris* had a closer relation to organisms belonging to the class Discosea (Visvesvara et al., 1993).

We described a new leptomyxid amoeba isolated from Mount Teide in the Canary Islands, Spain. The amoeba was denominated *Leptomyxa valladaresi* (de Obeso Fernandez del Valle et al., 2017).

5.1.2 Heterolobosea

Heterolobosea is a class created to join the groups Schizopirenida and Acrasid slime moulds (Page & Blanton, 1985). The two groups were first combined based on their eruptive lobopodia, discoidal organisation of the mitochondrial cristae and the

absence of a stacked Golgi apparatus. They are heterotrophic protozoans found worldwide in a wide range of habitats (Pánek & Čepicka, 2012).

Heterolobosean organisms present several different morphologies that include flagellates, amoeboflagellates, amoebae and even some slime moulds. Genetic studies of the 18S rDNA have proven the phylogenetic relationship between the very diverse members of the group (Brown et al., 2012; Roger et al., 1996). Heteroloboseans can present their rDNA in a circular plasmid in the mitochondria, as discovered first in *Naegleria gruberii* (Clark & Cross, 1987). One of the most important heteroloboseans is the amoeboflagellate *Naegleria fowlerii* since it is one of the causative agents of amoebic encephalitis. However, it is not the only heterolobosean linked to disease. *Vahlkampfia spp.* has been isolated from patients with keratitis in co-infection with *Hartmanella* and *Acanthamoeba* (Aitken et al., 1996; Alexandrakis et al., 1998; Niyyati et al., 2010).

Initially, all amoebic heteroloboseans that lacked a flagellate state were designated to the genus *Vahlkampfia*. This genus used to be defined by two main morphological characteristics: no flagellate state and cysts without pores. Also, they were recognised for their explosive locomotion, uninucleate cysts and closed mitosis. However, the study of the 18S rDNA showed that the genus was not monophyletic (Brown & De Jonckheere, 1999). Consequently, several genera were created to accommodate the different species. The new genera included *Allovahlkampfia*, *Paravahlkampfia*, *Neovahlkampfia*, *Fumarolamoeba* and *Solomistrus* (Brown & De Jonckheere, 1999; De Jonckheere, Johan F., Jun Murase, 2011; Walochnik & Mulec, 2009). From the different vahlkampfiids, the genus *Vahlkampfia* is the most diverse. The main reason for this, is that for some time, it was the sole genus of the family and encompassed all members that lacked a flagellate stage and cysts with pores (Pánek et al., 2017).

5.1.3 *Allovahlkampfia*

In 2009, *Allovahlkampfia* genus was created to fit an organism that did not fit anywhere else (Walochnik & Mulec, 2009). Since then, several new additions have been incorporated into *Allovahlkampfia*. There are no flagellates or amoeboflagellates in the genus. They are capable of producing simple cysts without operculae. *Allovahlkampfia* is a very diverse genus based on 18S rDNA sequences with some

organisms presenting inserts (Geisen et al., 2015). *Allovahlkampfia* is more closely related to Acrasid slime moulds than to other vahlkampfiids (Brown et al., 2012; Geisen et al., 2015).

We described a new hetereolobean amoeba isolated from the Glenfinnan viaduct in Scotland. The amoeba was denominated *Allovahlkampfia minuta* (de Obeso Fernandez del Valle & Maciver, 2017).

Chapter 6 *Acanthamoeba* encystment dynamics

6.1 Introduction

Acanthamoeba has a biphasic life cycle where it is capable of producing a latent protective cyst in adverse conditions to increase its survival rate. *Acanthamoeba* forms a double layered walled cyst consisting on an endocyst, an ectocyst and ostioles to monitor the environment and exit the cyst when conditions improve (Bowers & Korn, 1968; Chávez-Munguía et al., 2007; Weisman & Shaw, 1976).The cyst wall is 120-150 nm wide (Chávez-Munguía et al., 2007). The composition of the cyst varies depending on the species. However, studies performed by Neff et al. showed that the cyst contained 33% proteins, 4-6% lipids, 35% carbohydrates, 8% ash and 20% other unidentified materials (Neff et al., 1964b). Cellulose is one of the key components of the cyst wall comprising up to 10% of its dry weight (Blanton & Villemez, 1978; Tomlinson & Jones, 1962).

The first identified cause of encystation was starvation and lack of nutrients (Neff et al., 1964b). Nonetheless, other factors can induce encystment such as osmolarity, chemicals, temperature, pH and some bacteria (Brindley et al., 2009; Cordingley et al., 1996; El-Etr et al., 2009; Kilvington et al., 2008). In the environment, *Acanthamoeba* encysts synchronously (Neff et al., 1964b). However, once *Acanthamoeba* is cultured axenically, this characteristic is gradually lost, and the encystment process takes longer becomes less efficient and synchronous (Köhsler et al., 2008).

The cyst provides protection from most conditions that *Acanthamoeba* can encounter in the environment. In laboratory settings, it has been shown to be able to survive frozen for at least 25 years (Sriram et al., 2008). However, viable cysts have been recovered and grown from permafrost indicating they can survive for thousands of years (Shatilovich et al., 2009). *Acanthamoeba* cysts are capable of surviving at lower temperatures than other amoebae and are also able to survive up to 60˚C with moist heat for short periods (Biddick et al., 1984; Kilvington, 1989). Cysts have enhanced resistance to several physical, chemical and radiological factors including cellulases,

alcohol, hydrogen peroxide, gamma and UV irradiation (Aksozek et al., 2002). Cysts also provide a physical barrier capable of resisting biocides including many of the antimicrobial agents used for contact lens disinfection (Turner et al., 2000). Some of these disinfecting solutions induce encystment-like processes that quickly lead to the formation of pseudocysts (Kliescikova et al., 2011a). Pseudocyst formation is an alternate quick differentiation response from *Acanthamoeba* to acute stressors such as solvents. When *Acanthamoeba* encounters such stressors, pseudocysts appear in less than two hours (Kliescikova et al., 2011b). This resistance of the cysts changes depending on the encystment stimuli and the growth conditions of the trophozoites (Coulon et al., 2012).

6.1.1 Chapter objectives

Acanthamoeba differentiation rate and dynamics of different strains were compared to characterise the process. The use of conditions media (CM) was analysed to study its effect on encystment rate. Knowledge regarding the encystment process in *Acanthamoeba* will help understand the biology of this organism of great importance to the environment and human health. Eliminating the cyst might be the key to develop effective therapies against AK since cysts allow the persistence of the amoebae in the cornea (Nagington & Richards, 1976).

6.2 Materials and Methods

A diagram of the materials and methods used is shown in Figure 32. A more detailed explanation of the protocols and experiments performed can be found in Chapter 2.

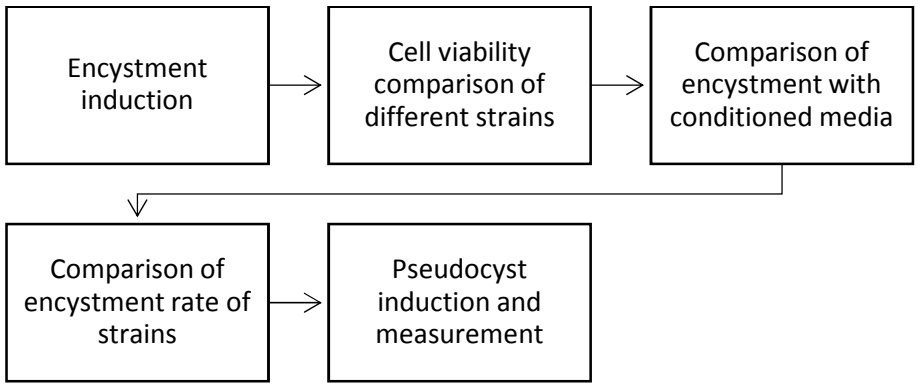


Figure 32. Diagram of methods used in Chapter 6 to characterise encystment dynamics.

6.3 Results

Different aspect of *Acanthamoeba* encystment were measured and compared. Using different conditions and strains, a picture of encystment rates was created to perform future experiments. Different cyst morphologies (and pseudocyst) can be seen in Figure 33.

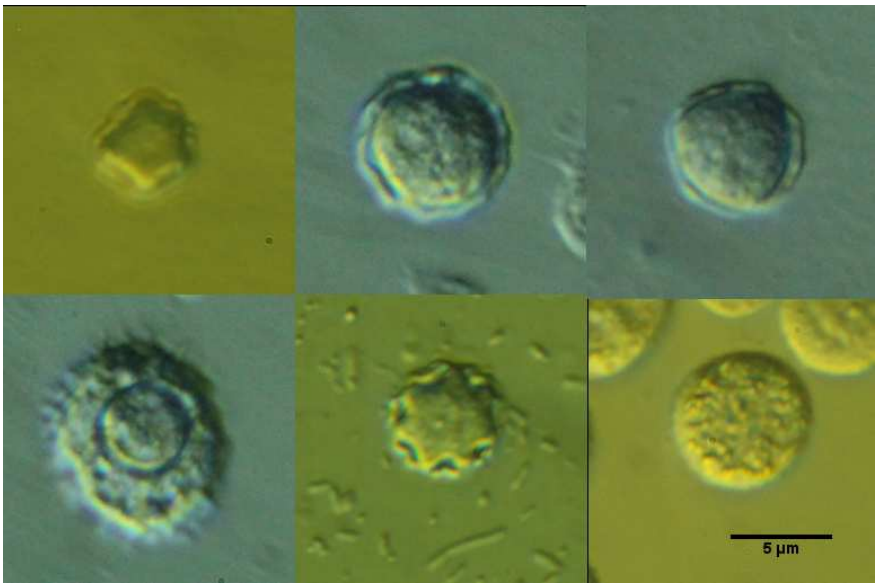


Figure 33. Different cyst morphologies. At the top, three different cyst morphologies from *A. castellanii* T4 genotype. On the bottom from left to right: A trophozoite "cannibalising" a cyst, a cyst surrounded by bacteria and a pseudocyst with a single-layered wall. The size bar represents 5 µm.

6.3.1 Cell viability after encystment

Trypan blue exclusion was used to verify cell viability after encystment. Cells that are alive do not take up the dye, while dead unviable cells stain in blue. The viability was measured for four different strains of *Acanthamoeba* (Neff, 53, 61 and 64). As shown in Figure 35, Neff strain encystment rate is significantly lower compared to other three strains. Strains more recently isolated showed a cyst viability between 29-42%, while Neff strain viability was under 10%. ANOVA analysis confirmed that there are significant differences between the viability of the strains (p value of 0.004). Additionally, two-tailed T-tests showed that there are significant differences between Neff and each of the other three strains. Strains 53, 61 and 64 did not show significant differences between them according to the T-test.

6.3.2 Observations of encystment rate over 96 hours

Additionally, the rate of encystment was measured from different strains to observe the time required for the process. Cyst of Neff strain and 47C started appearing around 12 hours after induction with NEM. The encystment rate of the four strains of *Acanthamoeba* was measured through cell count for a week. The proportion of cyst and trophozoites in cultures during encystment of newly isolated strain 53 (the most recently isolated strain) changed slower compared to the other strains as shown in Figure 35 and Figure 36c. In addition, Neff strain's encystment rate was compared to 47C. After selection of viable cysts, Neff strains encystment was accelerated as shown in Figure 36a and Figure 37. The results were corroborated through direct cell count and spectrofluorometry. The encystment rate appears to be strain dependant and not dependent on the time of axenic growth, as strain 53 shows the slowest encystment being the most recently isolated strain. An example of encysting strain 53 is found in Figure 34.

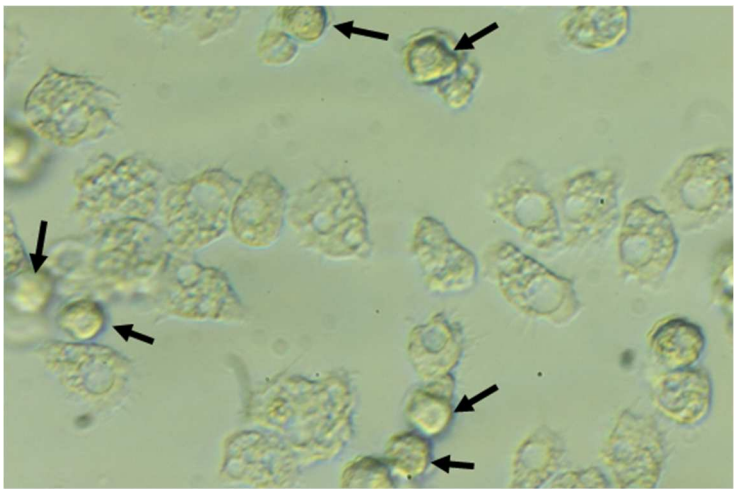


Figure 34. Microscopy image of strain 53 encystment after 36 hours for direct cell count. The arrows are pointing towards cysts, while the other cells are trophozoites.

6.3.3 Comparison of encystment with fresh and conditioned media of several strains

Since it has been observed that axenic cultures change their encystment dynamics, conditioned media was used in four different strains (Neff, 53, 61 and 64) to observe its effect on encystment. Conditioned media was previously obtained from encysting cultures. Independent T-tests for each strain were performed to compare the effects of conditioned media in each strain. Results showed significant differences on the encystment process when using conditioned media on two of the four strains (Figure 35 and Figure 36b and d). Strains 61 and 64 showed attenuated encystment when cultured with conditioned media. Strains Neff and 53 did not show significant differences (Figure 35).

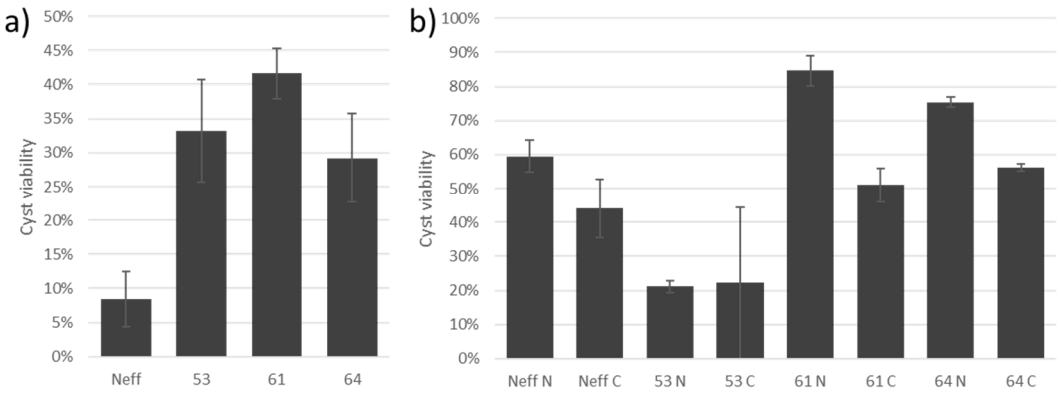


Figure 35. Encystment rate under different conditions. a) Viability of cyst after encystment from different strains after 96 hours in encystment media. The error bars represent the standard error of the mean. b) Comparison of encystment between four different strains with regular and conditioned media. The results show the percentage of cysts in comparison to trophozoites found after 96 hours in encystment media. The strains are Neff, 53, 61 and 64. The N after the strain number represents NEM media while the C refers to conditioned media. The error bars represent the standard error of the mean. Each strain included one picture from six samples with an average of 82.75 cells per picture.

6.3.4 Spectrophotometry to measure encystment

Spectrophotometry was used as a less biased alternative to the direct cell count used in most experiments. Samples were stained with Congo red, which binds to cellulose and is found in cysts but is lacking in trophozoites. Stained sampled were washed before measuring absorbance at a wavelength of 495 nm. Neff strain cultures were used after incubation with AX2, NEM and CM. Cellulose levels in the samples were measured via spectrophotometry and showed increased absorbance from encysting cultures. However, after two days the absorption in the cysts decreased. Use of stains such as Congo red after two days produced loss of sample due to the handling. Increased handling steps amplified the possibility of sample loss since cysts do not adhere to the bottom of the tube. The loss is reflected in Figure 36d where intensity declined after 48 hours.

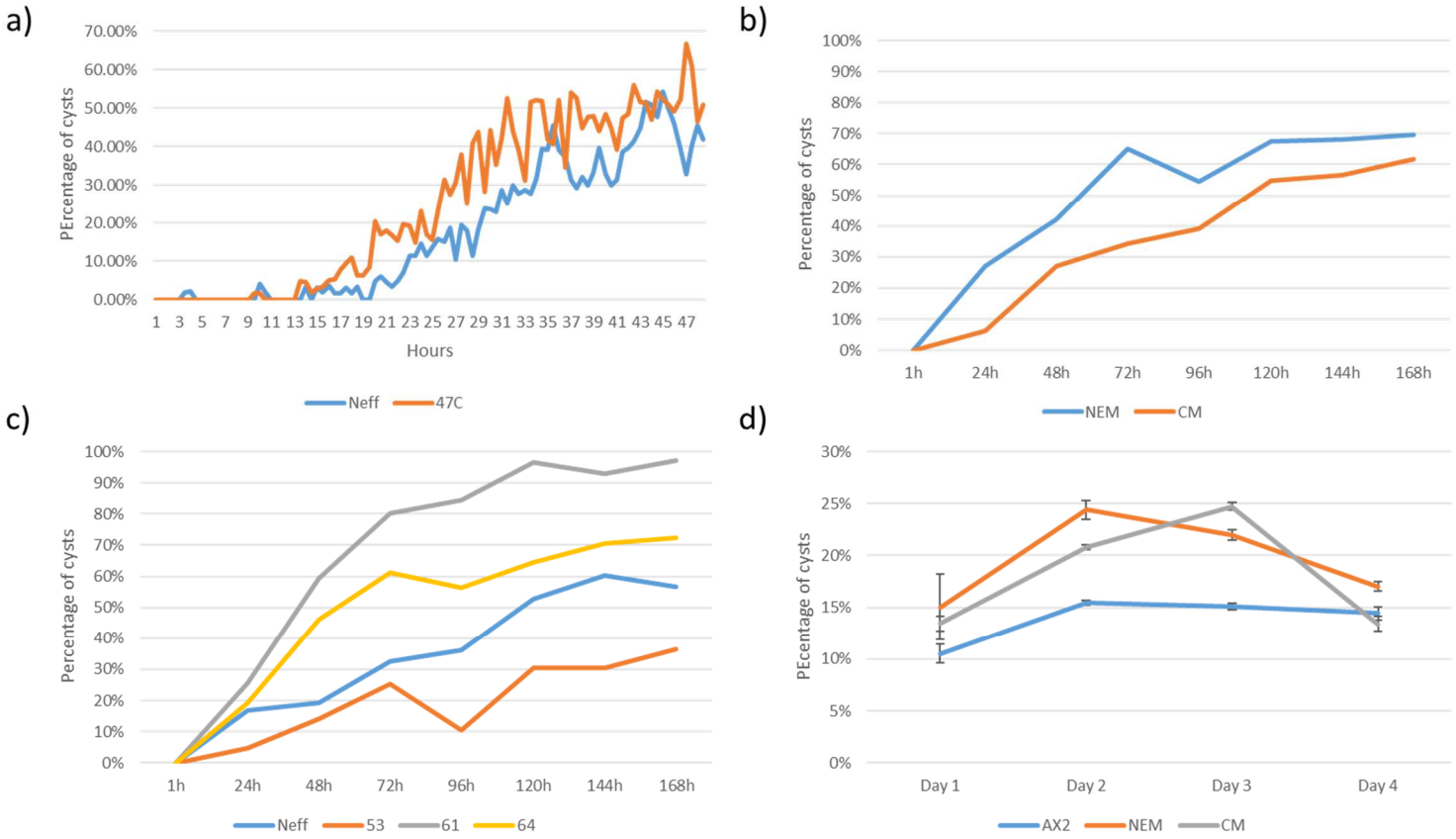


Figure 36. *Acanthamoeba* encystment dynamics. a) Encystment of Neff strain and 47C after 48 hours from photographic evidence and direct cell count every 30 minutes. b) Comparison over 168 hours of encystment of Neff strain encystment in NEM and CM from photographic evidence and direct cell count every 24 hours. c) Comparison of encystment over 168 hours of four different strains (Neff, 53, 61 and 64) from photographic evidence and direct cell count every 24 hours. d) Results over four days of encystment measured with spectrophotometry using Congo red as a stain. Trophozoites in AX2 were used as control and were compared every 24 hours. The error bars represent the SEM.

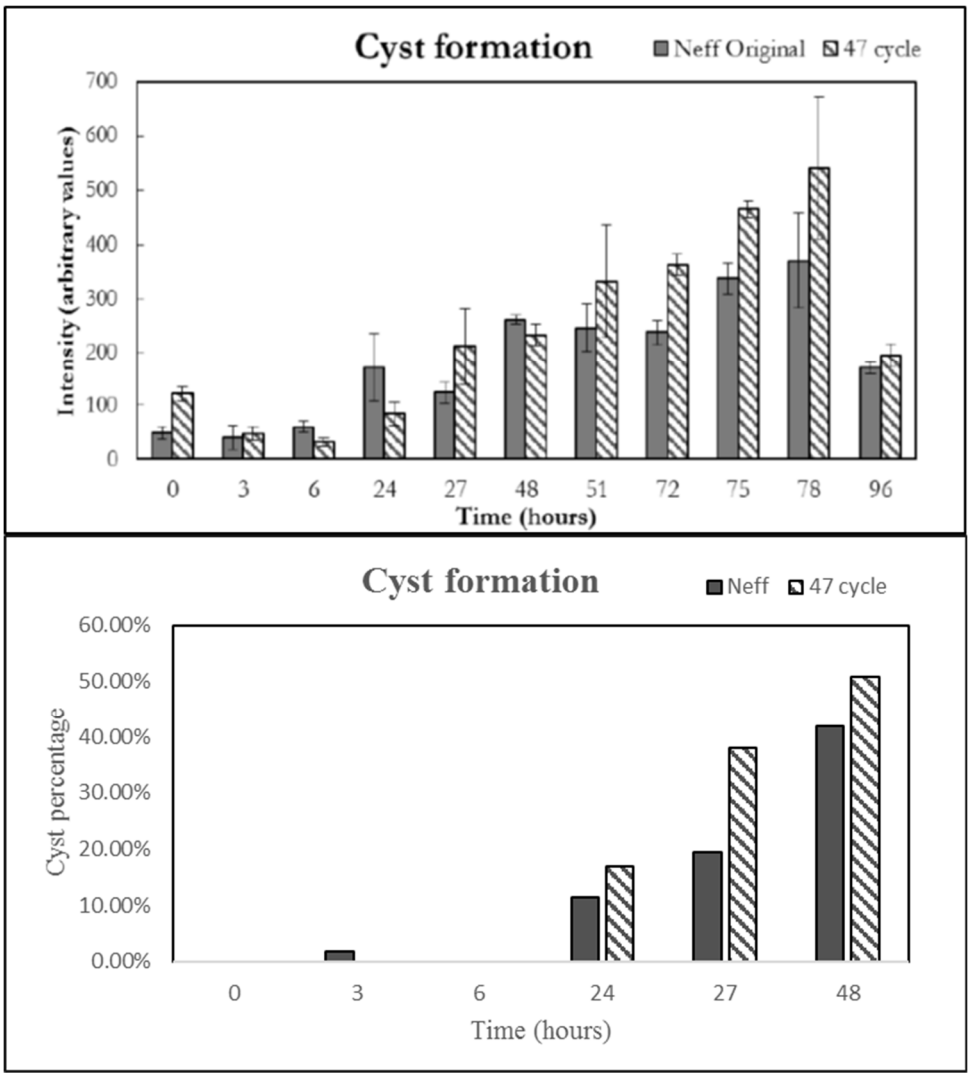


Figure 37. Encystment comparison between Neff and 47C. At the top, results from spectrofluorometry taken over a 96 hour period. The error bars show standard error of the mean. The graph was obtained from Katy McCalister's dissertation. At the bottom, results obtained from direct cell count over a 48 hour period taken every 30 minutes and selecting the same time points shown in the top graph. No error bars are shown since only one measurement was taken at each time point.

6.3.5 Formation rate of pseudocysts

Pseudocyst differentiation rate was also researched using spectrofluorometry. These experiments were supervised for this project, as they were performed by Katy McCallister.The process of pseudocyst differentaiton was triggered by the presence of methanol. Fluorescence signal increased for the first 90 minutes. After this period, the intensity started to decrease as shown in Figure 38. Results show a quick

response and differentiation for pseudocyst formation and probable quick production of cellulose, unlike encystment.

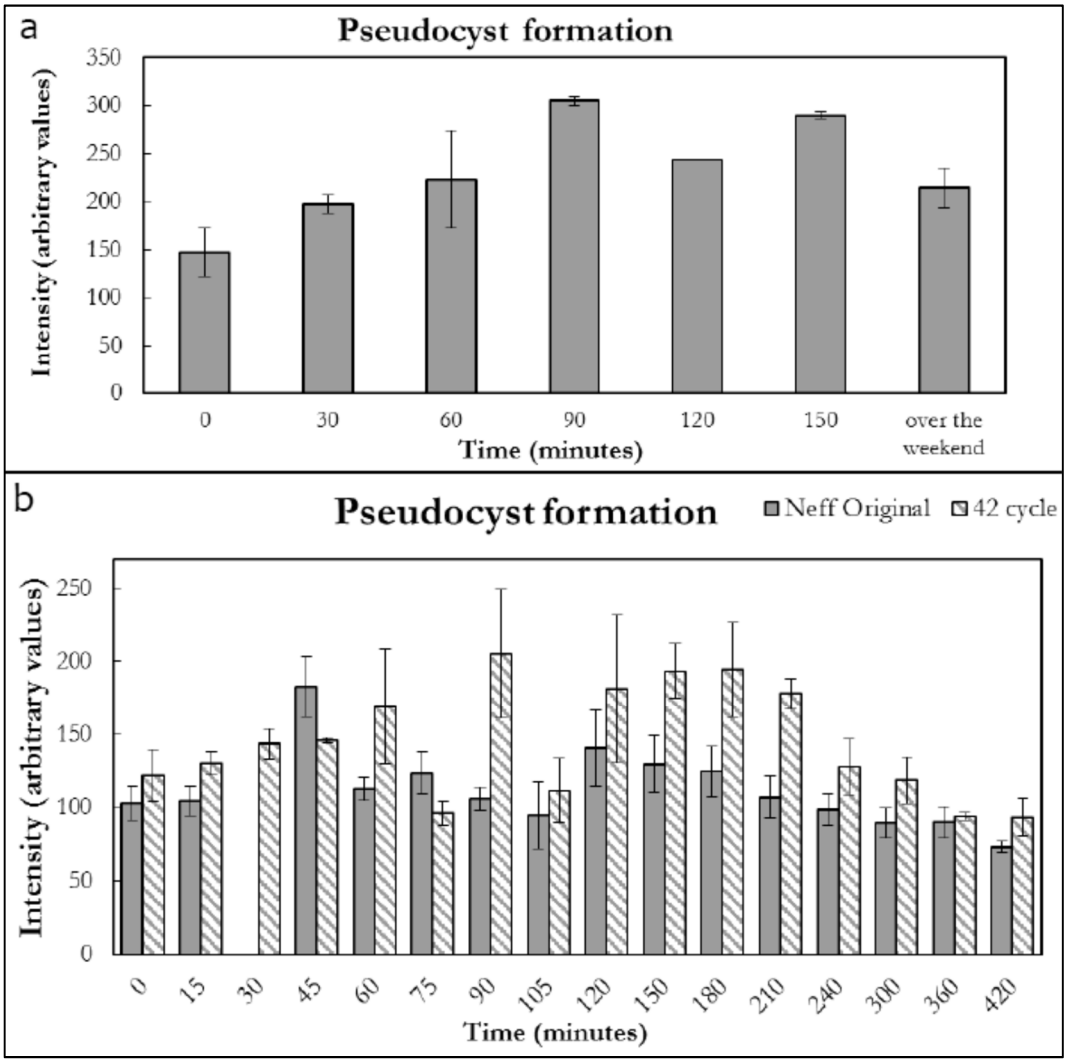


Figure 38. Pseudocyst formation. Obtained from Katy McCalister's dissertation. A) Pseudocyst formation starts and fluorescence increases over the first 90 minutes in methanol and then decreases. B) Pseudocyst formation comparison of Neff strain to 42C, showing a higher rate for the 42C strain. Error bars show standard error of the mean.

6.4 Discussion

After studying encystment dynamics, asynchronous encystment as described by Köhsler was observed with similar results for the different strains (Köhsler et al., 2008). After 24 hours less than 30% of the cells had encysted, and after 72 hours, encystment had not surpassed 70%. The asynchronous encystment on axenic

cultures and the realisation of varying rates of encystment in different strains are a challenge to understand the biology of the amoeba (Neff et al., 1964b). Comparisons between species and encystment conditions are still needed since cyst resistance can vary from one organism to the other and depend on the situation (Coulon et al., 2012). The effects of different encystment media and environmental conditions have been compared (Aqeel et al., 2013). Furthermore, encystment rate has been linked to the time the strain has been growing axenically (Köhsler et al., 2008). However, results of two T4 strains in these study (Neff and 53) do not support these findings as strain 53 showed a slower encystment.

Once the encystment rate for different strains was estimated, we decided to research cell viability after encystment. Neff strain viability has decreased after more than 50 years of in vitro culture. It has been suggested that the loss in viability occurs in part due to cannibalism (Maciver et al., 2015b). Neff strain has lost a significant part of its capacity to encyst. However, even with an attenuated capacity to produce cysts, Neff strain can regain some of this capacity through a selection of viable cysts using SDS (Maciver et al., 2015a). This selection also accelerated the encystment rate of the strain as shown in Figure 36a. Figure 36b and d show how conditioned media slowed the encystment in two of the four strains. This might indicate that there are no signals from *Acanthamoeba* to induce encystment to other amoebae. However, there might be changes in the environment that indicate other cells to delay encystment. Adaptations to these conditions might have led to cannibalism in the Neff strain (Maciver et al., 2015b). The viability of the cysts of all the strains was below 50%. With such a low cyst viability, it is possible that cells receive information from conditioned media to delay encystment as much as possible to increase survival.

Finally, we researched pseudocyst formation rate. Even though *Acanthamoeba* is considered to have a biphasic life cycle, we know that this is not precise as *Acanthamoeba* has trophozoites, cysts and is capable of forming pseudocysts in certain conditions (Kliescikova et al., 2011a, 2011b). The idea that encystment might only be induced axenically in some biphasic organisms might be wrong (Chávez-Munguía et al., 2007). Pseudocyst formation is a quick response to stressors such as solvents (Kliescikova et al., 2011b). This differentiation process increases over the first 90 minutes and then decreases slowly. Afterward, the fluorescence intensity stabilises and remains relatively similar for at least three days. Pseudocyst

differentiation is a quick response to sudden changes in the environment, for which encystment is not a viable option. It might also be useful to perform further research of *A. pyriformis* as it is the one member of the genus with a different life cycle (Tice et al., 2016).

Even though they are simple and accessible, microscopic cell counts are labour intensive and tend to have high variance (Lloyd, 2014). Therefore, different alternatives developed to study encystment. Normally, the progress of encystment can be measured by cellulose estimation (Griffiths & Hughes, 1969). For spectrophotometry, the use of 495 nm wavelength was adequate when staining with Congo red (Iwunze, 2010). For more precise measurement, fluorometry was measured with calcofluor white at an excitement length of 355 nm for excitation and 442 nm for emission which proved adequate (McCalister, 2015). Spectrophotometry proved useful in the first 48 hours after adding the encystment media. However, the value of the technique is lessened after two days as technical challenges arise. An increase in the proportion of cysts leads to the inability of the sample to adhere to the bottom of the tube after centrifugation increasing the probability of some sample to be lost with increased handling. Similar challenges were encountered using spectrofluorometry. The high sensitivity of the technique made results highly variable as some readings decrease and then increased from one time point to the next. Both techniques still proved valuable. Increasing the number of data points collected and developing a way to adhere the cyst to the tubes might provide a more precise and less labour intensive alternative (Griffiths & Hughes, 1969). Access to more advanced cell-counting techniques such as flow cytometry might also help provide more data regarding encystment and pseudocyst formation.

Knowledge of *Acanthamoeba* encystment is important for the understanding of the biology of this ecologically and medically important organism (Aqeel et al., 2013). Cysts can differ in morphology, resistance and viability. Understanding of the differences and similarities of the process of the three morphogroups might provide valuable information (Visvesvara, 1991). It could also help develop therapies for AK because cysts can lead to recurrent infection as they can resist many of the current therapeutic options (Larkin et al., 1992; Lorenzo-Morales et al., 2013; Turner et al., 2004). Also, *Acanthamoeba* serves as a vector for several pathogenic bacteria such as *L. pneumophila, S. aureus* and *C. pneumonia* (Essig et al., 1997; Huws et al., 2008;

Kilvington & Price, 1990). In many cases, these bacteria would be unable to persist in the environment without using amoebae as hosts. The cyst provides a haven for bacteria in several otherwise inhospitable environments. A higher understanding of the encystment process is important for future ecological and health studies.

Chapter 7 mRNAseq of encystment

7.1 Introduction

Gene expression patterns have been investigated to understand the life cycle of a number of encysting protozoa (Moon et al., 2011). Microarray analysis for encystation of *Acanthamoeba* and the intestinal amoeba *Entamoeba hystolytica* have been performed (De Cádiz et al., 2013; Ehrenkaufer et al., 2007; Moon et al., 2011). Differentially expressed (DE) genes have also been identified from *A. castellanii* using random primers and RT-PCR (Moon et al., 2007). In addition, Serial Analysis of Gene Expression (SAGE) has been used to analyse the transcriptome of the flagellated parasite *Giardia lambia* (Birkeland et al., 2010). Blocking encystation might be the key to avoid persistence of parasitic protozoans (Makioka et al., 2000, 2001, 2002).

Encystment consists of a two-step process that begins with autophagy and degradation of proteins and is followed by the expression of cyst-specific genes that rebuild the cell into a cyst as seen in Figure 39 (Leitsch et al., 2010). Gene expression during *Acanthamoeba* differentiation has been studied including factors involved in autophagy (Moon et al., 2009, 2012; Song et al., 2012), cellulose synthesis (Potter & Weisman, 1972), proteases (Leitsch et al., 2010; Moon et al., 2008b), and cyst wall proteins (Hirukawa et al., 1998; Rubin et al., 1976). Additionally, rRNA proteins are downregulated during encystment (Detke & Paule, 1975; Schulze & Jantzen, 1982; Stevens & Pachler, 1973).

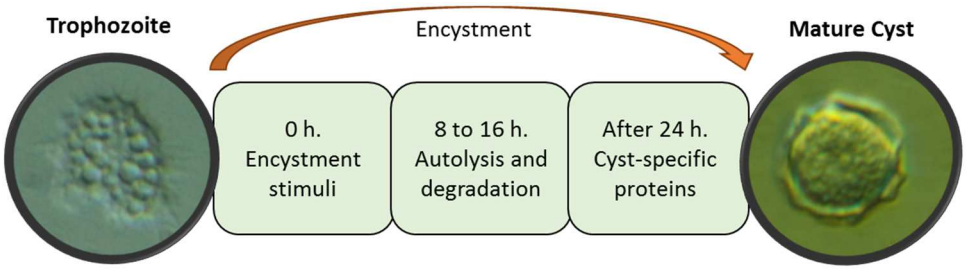


Figure 39. Diagram with simplified encystment process of *Acanthamoeba*.

A cyst specific 21kDA protein (CSP21) has been identified as part of the cyst wall (Hirukawa et al., 1998). CSP21 is regulated by mRNA expression being tightly

repressed during the trophozoite stage and highly induced during the early stages of encystment (Chen et al., 2004).

7.1.1 Chapter objectives

Previously, 12,544 genes of *Acanthamoeba* have been investigated after three days of encystation using microarrays (Moon et al., 2011). For this study, mRNA sequencing was performed to identify encystment factors using the reference genome recently published (Clarke et al., 2013). Additionally, we tried to identify different pathways involved and corroborate previously identified encystment factors.

7.2 Materials and Methods

A diagram of the materials and methods used is shown in Figure 40. A more detailed explanation of the protocols and experiments performed can be found in Chapter 2.

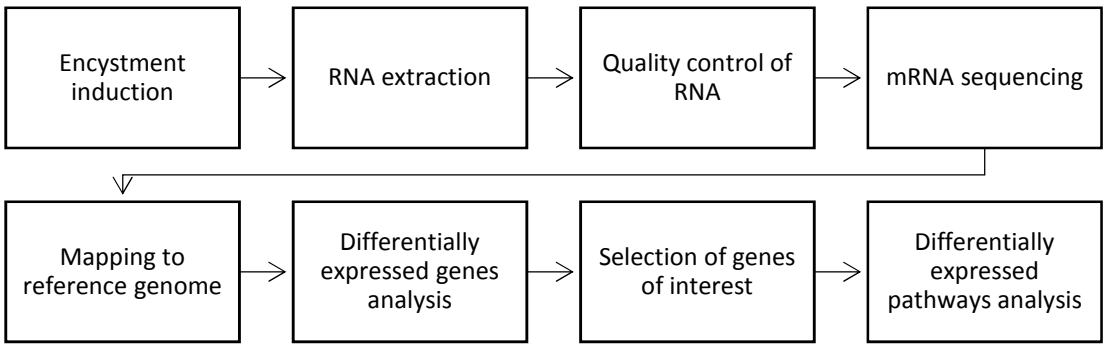


Figure 40. Diagram of methods used in Chapter 7 to identify factors involved during encystment through RNA sequencing.

7.3 Results

The genetic factors involved in encystation were studied though mRNA sequencing. The sequencing was performed using strain 53 from genotype T4 as it produced mostly viable cysts (Chapter 6). Four treatments (trophozoites, 24 hours after induction, 48 hours and 72 hours) were sequenced with three replicates each. The RNA from the twelve cultures was purified. Before sequencing, the quality of the

samples was verified using a QUBIT fluorometer and agarose electrophoresis. Good quality samples could be identified through gels when the two bands belonging to the ribosomal RNA can be clearly observed. Once quality was approved, samples were sent for sequencing to Edinburgh Genomics. Example samples of the agarose gels are shown in Figure 41.

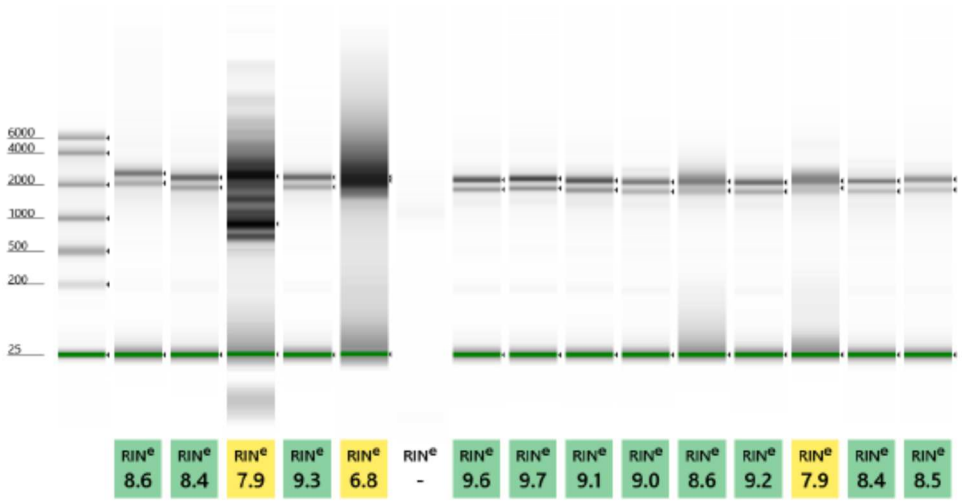


Figure 41 Sample figure from Edinburgh Genomics showing acceptable quality of RNA samples for sequencing. A molecular size ladder is situated in the left. In the middle, the image of the agarose gel where ribosomal RNA can be observed in most cases. In the bottom, the RNA Integrity Number (RIN) is shown. One of the samples was lost. Therefore it cannot be seen in the gel, and the RIN number is missing.

7.3.1 Data visualisation of data for quality control

Once the raw data from the mRNAseq was received, new steps to ensure quality control were taken. The quality of the data was assessed using FASTQC software that allowed for quick analysis of each sample. This step was performed twice as Edinburgh Genomics performs their own quality control assessment before data delivery.

Before processing and analysis of the results, visualisation of the data is recommended as a final step of quality control. The different visualisation steps showed that the data was reliable and that there were no outlier samples. First, a multidimensional scaling (MDS) plot was created using EdgeR. The plot Figure 42) shows four different groupings that belong to the four timepoints where none of the samples are outliers.

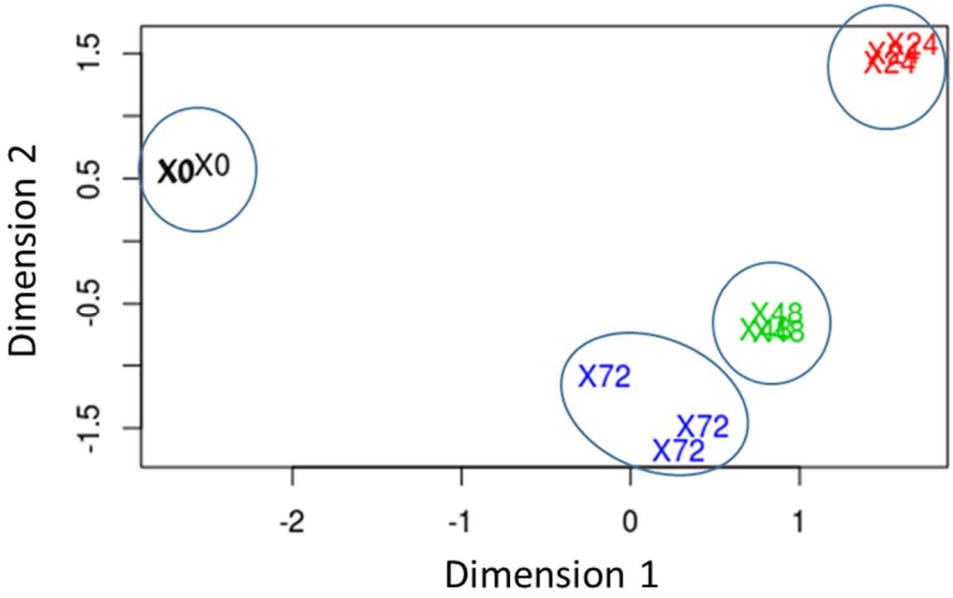


Figure 42. Multidimensional scaling plot. The plot shows the distribution of the samples after the sample pairing with added circles to encompass the four different timepoints (0, 24, 48 and 72 hours). The axes represent the dimensions assigned by R while creating the plot, but do not represent specific units.

The last visualisation step is the observation of dispersion and distribution of the data. Pairwise comparisons were performed between the four treatments leading to six different comparisons. According to the EdgeR manual, a biological coefficient of variation of 0.1 is to be expected for data from genetically identical model organisms, which is the closest description to the data presented as all the samples came from the same strain (Robinson et al., 2009). Dispersion plots for all comparisons gave trended values between 0.05 and 0.25 as seen in Figure 43. Figure 44 presents volcano plots for the six different comparisons that highlight differentially expressed genes.

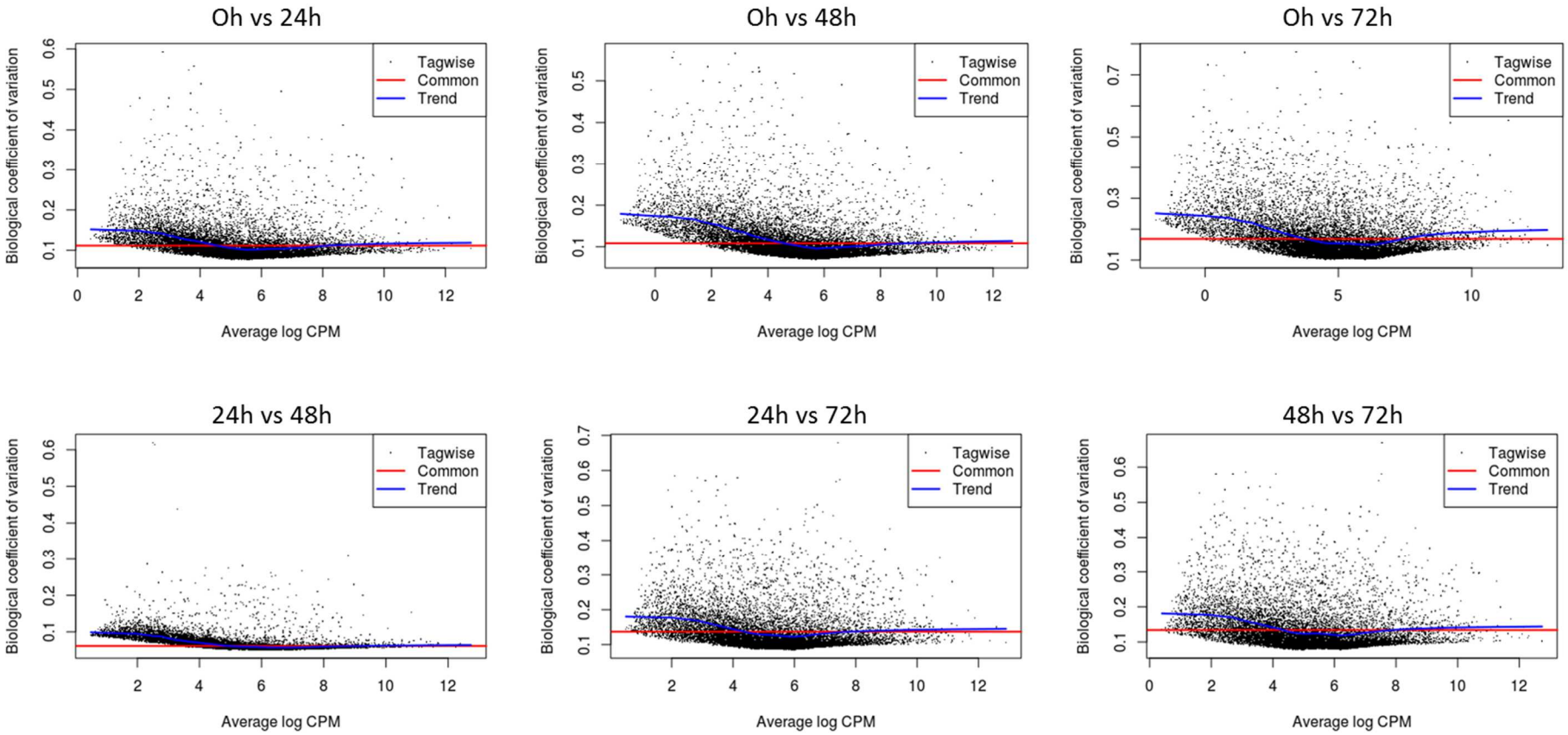


Figure 43. Dispersion plots for the six different comparisons of the four treatments. The plots show tag-wise dispersion trended with values around 0.1 for the trended Biological coefficient of variation.

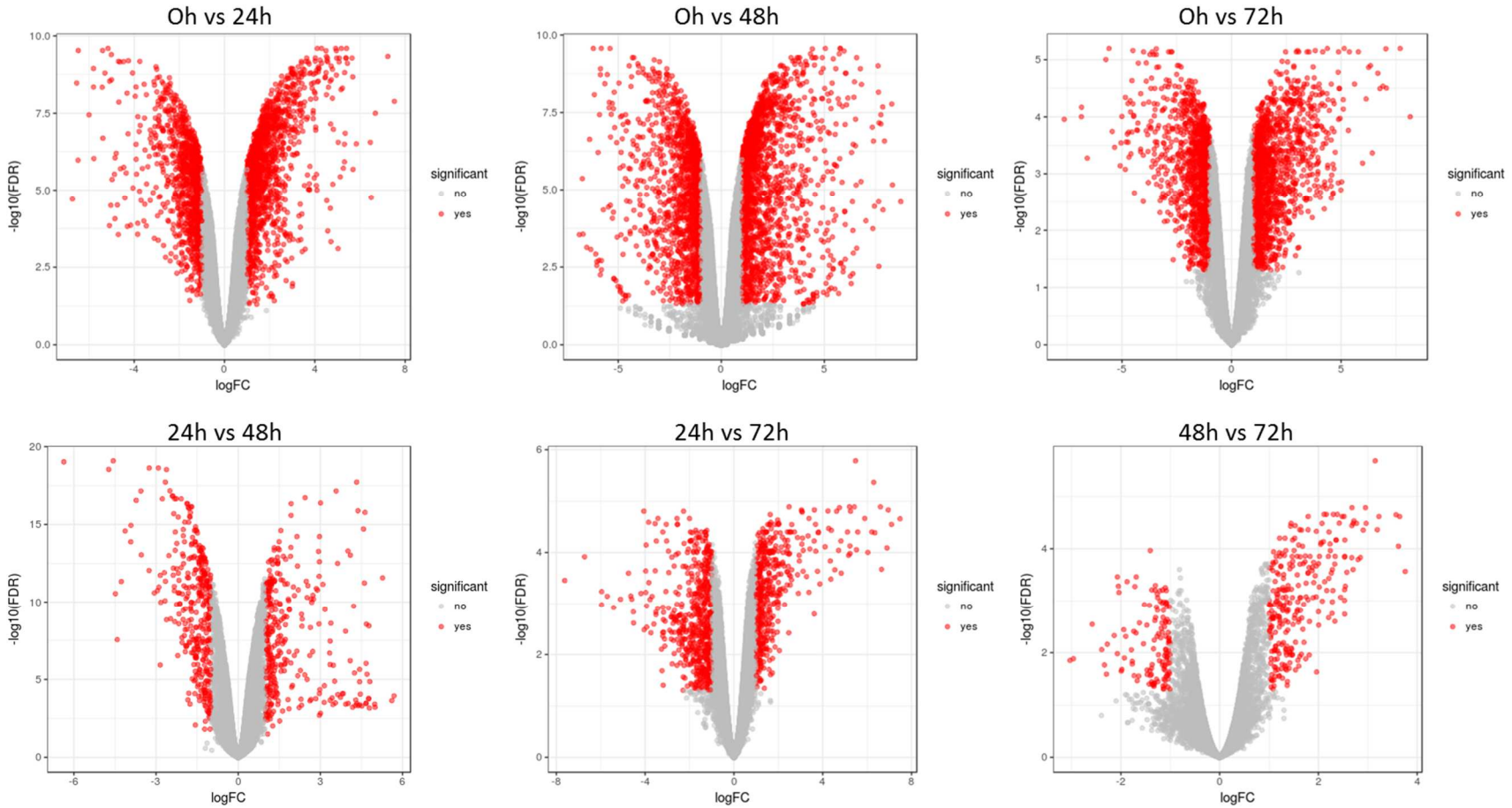


Figure 44. Volcano plots for the six different comparisons. The DE genes are represented in red. In the X-axis, LogFC (base 2) of 1 was used as criteria representing a fold change of 2. In the Y-axis, a Log10(FDR) just above 1 was used representing an FDR threshold of 0.05.

7.3.2 Identification of differentially expressed (DE) genes

After quality control assessment, the raw data was aligned to the reference genome, and reads were counted. Once the reads were mapped into the genome, the genetic factors involved in encystation were researched through differential expression analysis using EdgeR. The number of DE genes along the six different comparisons using a criterion based on a fold change (FC) of 2 (Log2 of 1, referred forward as LogFC) and a false discovery rate (FDR) threshold of 0.05 is shown in Table 15. The comparison with the highest amount of DE genes was between 0 and 24 hours. The difference between 0 hours and the other treatments in the number of DE genes was considerably higher than the comparisons with the samples already going through differentiation. For example, there are only 31 DE genes with a LogFC over 2 and none over 5 between 48 and 72 hours. Tables with the top 50 DE genes for each comparison can be found in Appendix 5. For every comparison, there were more downregulated than upregulated genes among the top 50 DE genes. In the case of the comparison between 0 and 24 hours, 12 genes were upregulated, while 38 were downregulated. Figure 47 shows the 12 genes that are upregulated after 24 hours.

The expression and distribution of all the genes, with the DE genes highlighted, can be observed in the volcano plots in Figure 44.

	0 hours	**24 hours**	**48 hours**	**72 hours**
0 hours		3474	3337	2820
24 hours	3474		881	1383
48 hours	3337	881		475
72 hours	2820	1383	475	

Table 15. Number of DE genes between the different time points with a fold change (FC) of at least 2 and a maximum FDR of 0.05.

CSP21 was used as a positive control to make sure that the results and the expression profile fitted with expected results. The results for CSP21 expression across the 12 samples are shown in Figure 45. Expression of CSP21 was close to zero in trophozoites but was upregulated in the first 24 hours of encystment. The number of transcripts decreased afterwards, nearing zero after 72 hours.

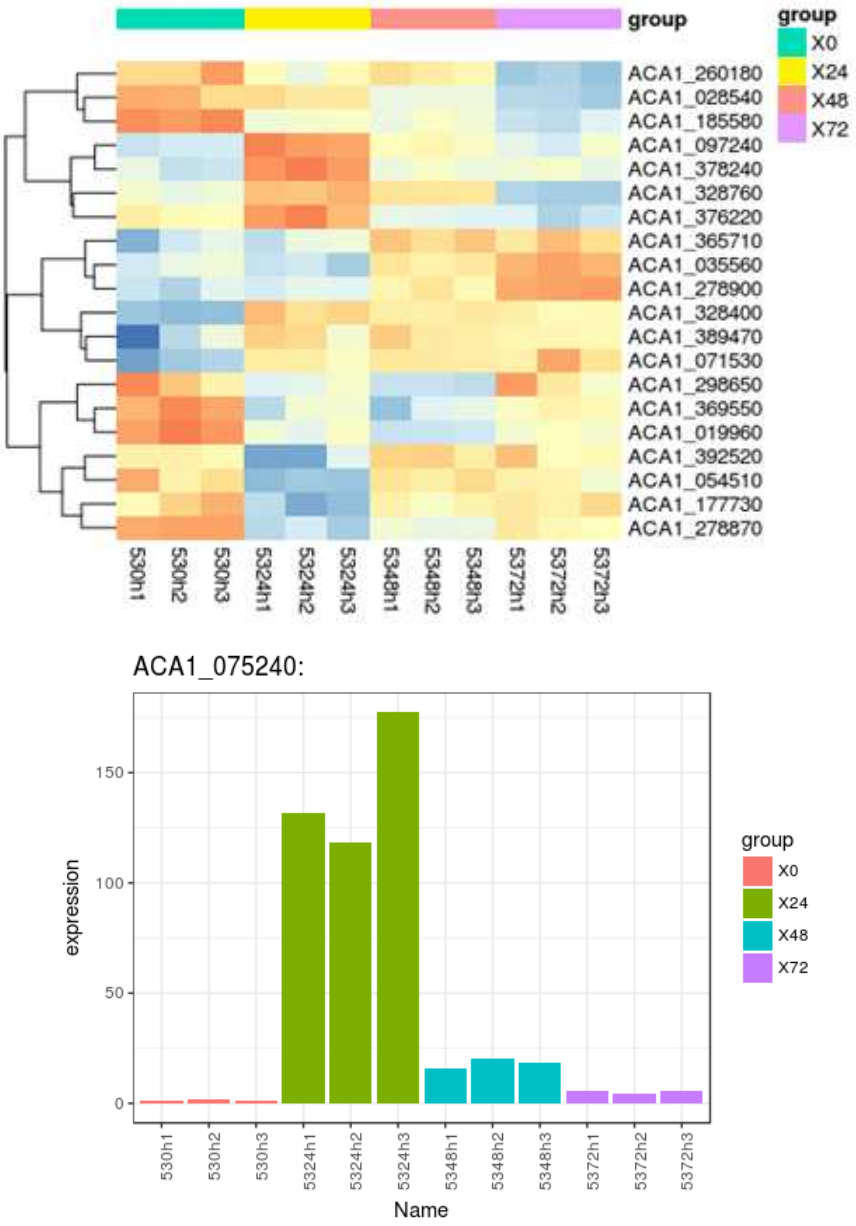


Figure 45. Heatmap of differential expression and expression bar chart of CSP21 as a control. On top, a heatmap of the top 20 differentially expressed genes (obtained from the EdgeR analysis for DE genes) from the comparison of the four different treatments. Warm colours (closer to red) represent upregulated genes, while cool colours (closer to blue) represent downregulated genes. An X and the number of hours of each treatment represent the groups. On the bottom, expression of CSP21 across the different samples and treatments. The groups are labelled as X0, X24, X48 and X72 representing the four different treatments, relating the number to the number of hours. The measurement of expression is in CPM.

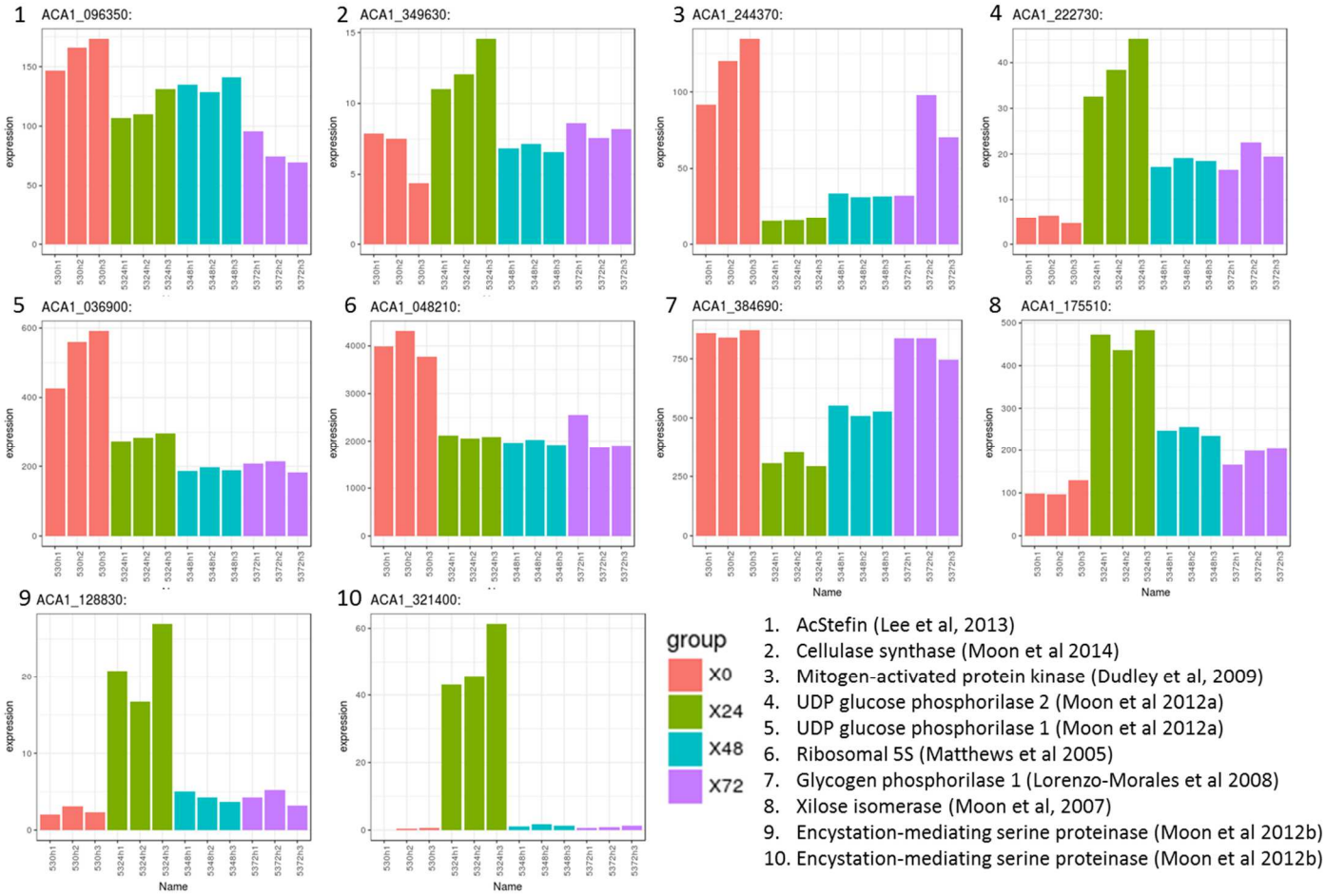
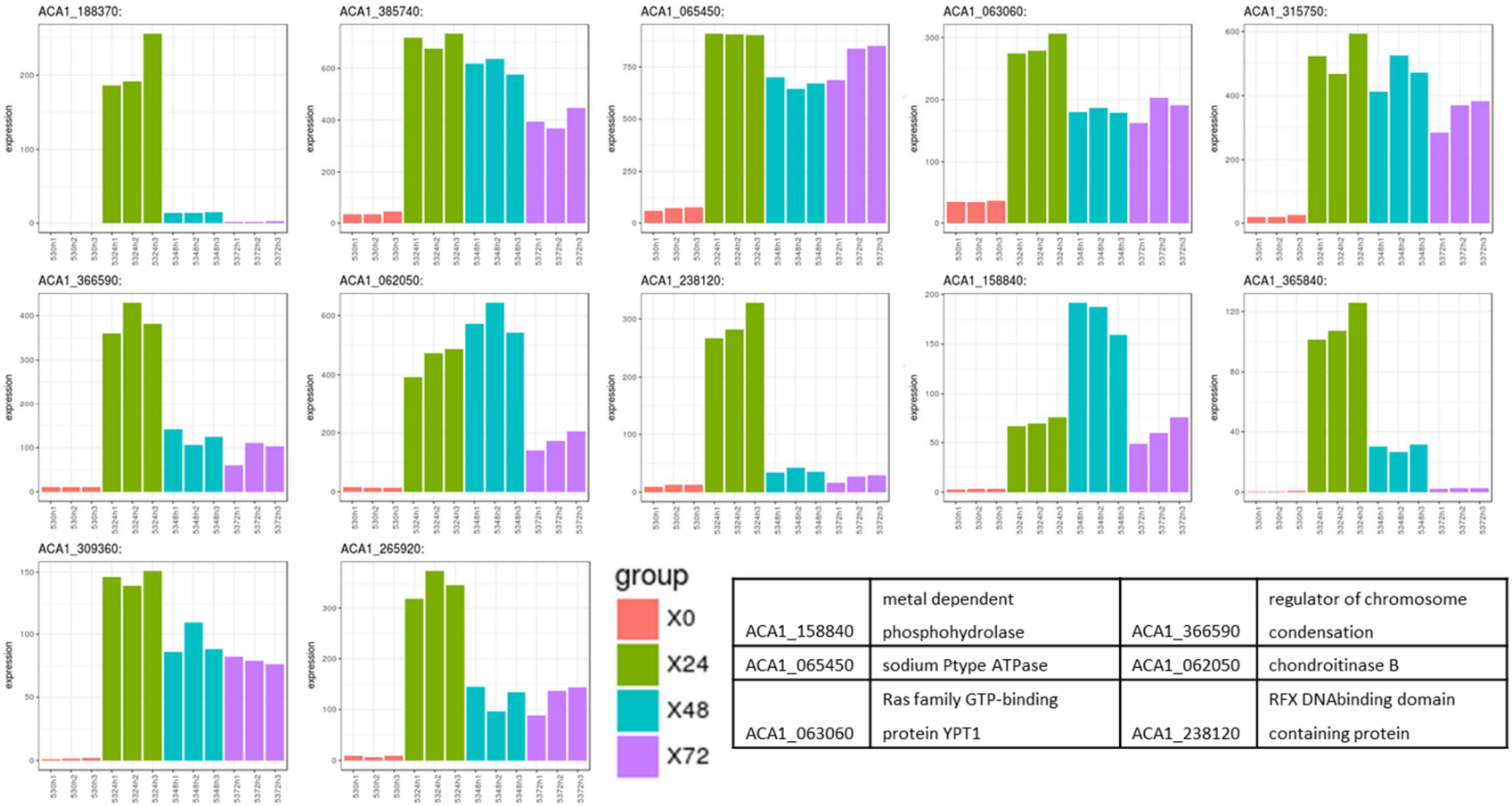


Figure 46. Expression of 10 different selected DE genes during encystation according to the literature. The expression is presented in CPM. Each bar chart shows the four different treatments (groups are represented with an X and the number of hours) with their three samples.



ACA1_158840	metal dependent phosphohydrolase	ACA1_366590	regulator of chromosome condensation
ACA1_065450	sodium Ptype ATPase	ACA1_062050	chondroitinase B
ACA1_063060	Ras family GTP-binding protein YPT1	ACA1_238120	RFX DNAbinding domain containing protein

Figure 47**. Upregulated genes after 24 hours of encystment from the TOP 50 DE between trophozoites and 24 h treatment. The graphs show the expression during the 72 h. The genes are identified by the gene_id at the top of each graph (genes not mentioned in the box belong to hypothetical proteins) Expression is measured in CPM.**

Upregulated after 24 hours	Upregulated after 48 hours	Upregulated after 72 hours
acan00592 alpha-Linolenic acid metabolism	acan00071 Fatty acid degradation	acan04144 Endocytosis
acan04144 Endocytosis	acan04144 Endocytosis	acan04141 Protein processing in endoplasmic reticulum
acan00563 GPI-anchor biosynthesis	acan00592 alpha-Linolenic acid metabolism	acan00562 Inositol phosphate metabolism
acan00770 Pantothenate and CoA biosynthesis	acan00562 Inositol phosphate metabolism	acan00592 alpha-Linolenic acid metabolism
acan00562 Inositol phosphate metabolism	acan04120 Ubiquitin mediated proteolysis	acan00563 GPI-anchor biosynthesis
acan00071 Fatty acid degradation	acan00563 GPI-anchor biosynthesis	acan00071 Fatty acid degradation
acan00531 Glycosaminoglycan degradation	acan00561 Glycerolipid metabolism	acan00561 Glycerolipid metabolism
acan00511 Other glycan degradation	acan04070 Phosphatidylinositol signalling system	acan04070 Phosphatidylinositol signalling system
acan00603 Glycosphingolipid biosynthesis	acan00471 D-Glutamine and D-glutamate metabolism	acan00511 Other glycan degradation
acan00430 Taurine and hypotaurine metabolism	acan00511 Other glycan degradation	acan00471 D-Glutamine and D-glutamate metabolism
acan04070 Phosphatidylinositol signalling system	acan00531 Glycosaminoglycan degradation	acan00531 Glycosaminoglycan degradation
acan00410 beta-Alanine metabolism		
acan00750 Vitamin B6 metabolism		
acan04120 Ubiquitin mediated proteolysis		

Only upregulated after 72 hours
Upregulated after 48 and 72 hours
Upregulated in the three timepoints
Only upregulated after 24 hours
Upregulated after 24 and 48 hours

Table 16. Upregulated pathways during encystment compared to trophozoites. The table shows the pathways that are upregulated with an FDR value under 0.01 in comparison to 0 hours. On the bottom right, the legend explains the colour code of which pathways are shared between treatments.

7.3.3 Comparison of DE pathways

Finally, the data was used to identify differentially expressed pathways to gain a larger understanding of gene expression and metabolism during encystment. The pathways from *Acanthamoeba* were obtained from KEGG Pathway database (Kanehisa et al., 2016, 2017; Kanehisa & Goto, 2000). There are 102 pathways recorded in the database. Between 0 and 24 hours, there were 14 pathways upregulated during encystment with an FDR value below 0.01. Upregulated pathways during the different timepoints of encystment are shown in Table 16. The most significant pathway differentially expressed was endocytosis. The expression profile of the endocytosis pathway can be found in Figure 48. In addition, a list with the DE pathways between 0 and 24 h can be found in Appendix 5.

	0h	24h	48h	72h
0h		76	77	51
24h	14		56	6
48h	11	12		3
72h	11	1	1	

Number of DE pathways

Number of pathways downregulated in relation to earlier timepoint

Table 17. Number of DE pathways. The table shows the number of pathways from the KEGG database that are differentially expressed and the number of pathways that are downregulated in relation to the earlier timepoint of the comparison. There are 102 pathways for *Acanthamoeba* in the KEGG database.

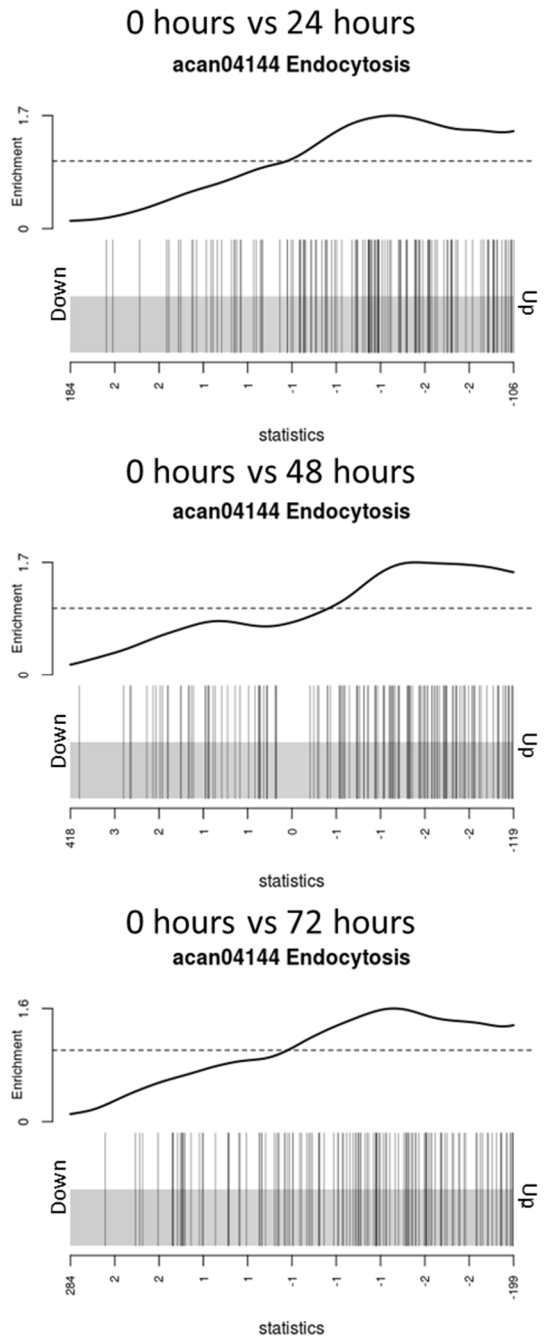


Figure 48. Differential expression of endocytosis pathway genes during encystment. Comparison of trophozoites against the three different timepoints. Downregulated genes during the timepoint are to the left, and upregulated genes are to the right. A numeric vector is generated (by GAGE software) and is represented in the X-axis as statistics for each comparison. Each vertical line represents one annotated gene within the pathway. The worm represents relative enrichment of the pathway. The dotted line represents an enrichment value of 1.

Pathway expression between 48 and 72 hours is similar. Only three pathways are differentially expressed between 48 and 72 hours with an FDR value under 0.01. These pathways are basal transcriptions factors (upregulated after 48 hours), caffeine metabolism (downregulated after 48 hours) and nucleotide excision repair (upregulated after 48 hours).

Several pathways related to molecule degradation are upregulated during encystment such as degradation of fatty acids, glycosaminoglycan and other polysaccharides. Ubiquitin-mediated proteolysis pathways found upregulated after 24 and 48 hours are related to the degradation of proteins required for cyst formation.

7.4 Discussion

There is a multitude of changes during the first 24 hours of encystment as it had the highest number of DE genes and pathways. In general, there are more upregulated genes and pathways during trophozoite stage than there are during encystment. The lower number of upregulated genes in encysting cells could indicate lower levels of expression systemically, as it differentiates into a latent stage. However, it has been reported that there is an increase in RNA, proteins and DNA at the beginning of the encystation process (Chagla & Griffiths, 1974).

Several encystment factors of *Acanthamoeba* have been identified, One such factor is CSP21, a cyst specific protein identified to occur exclusively during encystment (Hirukawa et al., 1998). CSP21 is recorded in GenBank and AmoebaDB with the gene_id ACA1_075240. The upregulation of CSP21 was confirmed through the expression profile as seen in Figure 45 and used as a control.

Figure 46 shows other previously reported proteins related to encystment that include cellulose synthase, xylose isomerase, glycan phosphorylases and proteases. Cellulose synthase is crucial in the formation of endocysts (Moon et al., 2012). Silencing cellulose synthase and xylose isomerase inhibits encystation (Aqeel et al., 2013). The results found for cellulose synthase and xylose isomerase indicate that both are upregulated during encystment. However, the expression of cellulose synthase was not significant under the criteria used presenting a LogFC below 2 and therefore was ignored for this study. Additionally, UDP glucose pyrophosphorylase 2

and glycogen phosphorylase have been related to encystment (Moon & Kong, 2012; Potter & Weisman, 1971; Weisman et al., 1969). Glycogen phosphorylase is the main mechanisms to maintain glucose levels required for encystment, and it required for the assembly of the endocyst wall (Lorenzo-Morales et al., 2008). However, only the gene for UDP glucose phosphorylase 2 (ACA1_222730) was upregulated during differentiation therefore it can be assumed it is partly responsible of maintaining glucose levels during the process. UDP glucose phosphorylase 1 and glycogen phosphorylase 1 were downregulated and do not seem to participate in the process. Also, the cysteine protease inhibitor AcStefin has been associated with full cyst formation (Lee et al., 2013). Nevertheless, results obtained from the analysis do not corroborate these findings, as AcStefin is not significantly upregulated.

In addition, proteases play an important role during encystation. Serine and cysteine proteases are important in the early stages of differentiation as they help break apart proteins before cyst wall formation (Leitsch et al., 2010; Moon et al., 2008b). Serine proteases were shown to have a 282 higher FC expression in cysts than in trophozoites using microarray analysis (Moon et al., 2011). Also, a serine protease from the subtilisin family was identified as a factor using RACE-PCR (Moon et al., 2007). In addition, a cyst specific cysteine protease degrades the mitochondria during differentiation (Moon et al., 2012). Additionally, AmoebaDB has two encystment mediating serine proteases that are upregulated after 24 hours on encysting media, and almost non-existent before or after. All the proteases related to encystment in the literature that could be linked to the dataset and genome annotation, were found to be upregulated.

Several of the proteins reported in the literature were not found in the dataset, therefore data could not be found. According to Moon, arginine methyltransferase 5 is an epigenetic regulator of encystment (Moon et al., 2016). However, the gene was not found in the available genome databases so it cannot be confirmed. Nevertheless, similar *Acanthamoeba* proteins such as arginine nMethyltransferase, or other proteins containing the domain found through AmoebaDB (ACA1_263190, ACA1_279220, ACA1_236480 and ACA1_236470), were not expressed at significantly different levels or were downregulated at a LogFC below 2. In addition, *A. castellanii* metacaspase protein is not annotated in the reference genome, therefore, could not

be analysed (Saheb et al., 2015). A more thorough and in-depth bioinformatics analysis should be capable of identifying some of these proteins in the genome.

Several new upregulated genes during encystment were identified and are shown in Figure 47. Such proteins include chondroitinase B, an ATPase, a regulator of chromosome condensation and a phosphohydrolase. Mainly, these proteins are related to protein degradation and energy utilisation. Chondroitinase (gene_ID ACA1_062050 in Figure 47) is upregulated during the first 48 hours of encystment. Chondroitinase helps in the degradation of chondroitin sulphate proteoglycans before the formation of the cyst. Chondroitin sulphate proteoglycans play a role in the adhesion to surfaces or host cells for other pathogenic protozoans such as *Plasmodium falciparum* and *Toxoplasma gondii* (Carruthers et al., 2000; Creasey et al., 2003). Upregulation of chondroitinase is related to the lack of adhesion of cysts since they do not require or are capable of adhering to surfaces or host cells. The lack of adhesion can help cysts move through aerosols increasing the likelihood of finding favourable conditions. Chondroitinase has been studied as a potential drug candidate (Kasinathan et al., 2016).

A big challenge and opportunity in working with mRNAseq from *Acanthamoeba* is the high number of proteins reported as hypothetical. From the top 50 DE genes between 0 and 24 hours, 19 were hypothetical proteins. Of the 19 hypothetical proteins, four had recognisable putative domains as shown in Table 18. Behera and Satpathy performed 2D SDS PAGE and identified several proteins as differentially expressed during encystment. These proteins included the “hypothetical” protein aCA1 and a “eukaryotic porin protein” (Behera & Satpathy, 2016).

Gene_id	LogFC	Protein Length	Putative domains (BLASTp)
ACA1_365840	-7.44646217	380	Endomucin superfamily
ACA1_227710	5.387266338	701	SMC_N superfamily
ACA1_282980	7.231880132	231	Ependymin superfamily
ACA1_186030	4.498676216	902	DNA polymerase III subunits gamma and tau

Table 18. Hypothetical proteins from the comparison between trophozoites and 24 encystation with a recognised domain obtained through BLASTp.

Once, the DE genes were analysed, an analysis was performed to look at the pathways involved. The KEGG database has 102 recorded pathways for *Acanthamoeba*. Large numbers of the pathways reported were DE, mainly during the

first 24 hours. However, most of the DE pathways were downregulated 24 hours after the encystment stimulus (over 81.5% of the DE pathways and 60.7% of the total pathways analysed compared to 24 hours). Only 14 pathways (13.7%) were found to be significantly upregulated during encystment after 24 hours. Of the upregulated pathways, several of them are related to molecule degradation required before cyst formation (Leitsch et al., 2010). Moon used KOG database using expressed sequence tags (ESTs) of differentially expressed genes to identify orthologous groups important in encystment (Moon et al., 2008a). However, the use of different databases and the limited number of fragments used do not make a comparison between the groups feasible.

Dudley et al. identified the mitogen-activated protein-kinase mediated pathways as being down-regulated during encystment (Dudley et al., 2009). Only one of the protein kinases recorded in AmoebaDB was found to be downregulated (Figure 46). However, the pathway is not recorded in the KEGG database, and could not be verified as a whole. Also, Moon et al (2011) reported another serine-threonine cyst specific protein kinase, was not found to be upregulated.

Endocytosis pathway is upregulated during the three timepoint of encystment as seen in Figure 48. *Acanthamoeba* Type-I metacaspase (Acmcp) is upregulated in encystment and is related to endocytosis and phagocytosis in *Acanthamoeba* (Saheb et al., 2014, 2015).

Study of the pathways and clarification of a larger number of pathways helps understand the systematics of *Acanthamoeba*. The understanding of the biology of *Acanthamoeba* at a higher level might help combat AK (Lorenzo-Morales et al., 2013). Some of the 14 upregulated pathways during encystment could be targeted to treat infection and deal with the persistence of the pathogen.

Understanding the molecular mechanisms of *Acanthamoeba* differentiation should help disrupt encystment, making it easier to treat infections. It could also help develop drugs against cyst-forming protozoa (Moon et al., 2007). The results presented in this study still need to be verified using qPCR. Additionally, mRNA sequencing analysis from other genotypes, pseudocyst differentiation, excystation and other time-points (8

hours and mature cysts after at least 96 hours) could prove valuable to have a better understanding of the process.

Chapter 8 *Acanthamoeba* enzymes

8.1 Introduction

Protozoans, such as *Acanthamoeba*, secrete different bioactive proteins that help modulate the interactions with other microorganisms (Bayer-Santos et al., 2013). *Acanthamoeba* produces several antimicrobials to target bacteria (Iqbal et al., 2014). *Acanthamoeba* produces different hydrolytic enzymes, such as proteases, whose main physiological purpose appears to be the degradation of substrates for nutrition purposes (Piña-Vázquez et al., 2012). *Acanthamoeba* feeds through phagocytosis and pinocytosis (Bowers & Olszewski, 1972). However, the mechanisms in which *Acanthamoeba* decides which process to undertake are poorly understood. In 1969, it was discovered that homogenates of *Acanthamoeba* presented ribonuclease, phosphatase, proteinase, α-glucosidase, β-N-acetylglucosaminidase, β-glucuronidase, amylase and peptidase activity (Muller, 1969; Rosenthal et al., 1969). *A. palestiniensis* presented a high level of α- and β- glucosidase activity (Lasman, 1975). Furthermore, Drozański identified bacteriolytic enzymes from *A. castellanii* (Drozanski, 1969, 1971; Drozański, 1978).

Proteases are some of the most commonly researched proteins from *Acanthamoeba*. Studies have shown that *Acanthamoeba* can produce proteases of varying molecular weight, including some of 12, 22, 24, 26, 33, 35, 42, 49, 55, 59, 66, 70, 80, 97, 100, 107, 130 and 230 kDa (Cao et al., 1998; Hadas & Mazur, 1993; Khan et al., 2000; Kim et al., 2003; Kong et al., 2000; Leher et al., 1998; Mitro et al., 1994; Na et al., 2001). Cysts do not show any extracellular protease activity (Mitro et al., 1994). Most of the molecular weights for the proteases mentioned before are only apparent as migration is delayed by interaction with the substrate during electrophoresis.

Acanthamoeba proteases play an important role in pathogenicity (Lorenzo-Morales et al., 2005b). This occurs as they increase cell permeability, are capable of disrupting cell monolayers and can degrade the human extracellular matrix (Alsam et al., 2005b; Piña-Vázquez et al., 2012; Sissons et al., 2006). In addition, some proteases are capable of producing cytotoxic effects (Cho et al., 2000). Furthermore, *Acanthamoeba*

produces proteases capable of degrading iron binding proteins, that helps keep free radicals loss and avoid tissue or cell degradation (Ramírez-Rico et al., 2015).

Previously, isoenzymes from *Acanthamoeba* have been used for classification (Jonckheere, 1983). However, comparisons between similar studies are complicated as enzyme production can be altered depending on the circumstances and according to the life cycle (Moon et al., 2008b). Nevertheless, enzyme activity comparison between strains and species could be used for ecological purposes (Costas & Griffiths, 1985). Proteases activity profile has been suggested as a way to differentiate between pathogenic and non-pathogenic *Acanthamoeba* strains (Khan et al., 2000). However it subsequently became clear that the situation is complex. Nevertheless, the secretion of lytic proteins mediates the lysis of microorganism and the invasion of tissue (Piña-Vázquez et al., 2012). Understanding the molecular processes by which *Acanthamoeba* feeds, and the enzymes it produces are of therapeutic importance.

8.1.1 Chapter objectives

Different secreted enzymes were researched with the goal of identifying potential antimicrobial agents and perform a simple characterization of such secretions.The characterization of isoenzymes can be of value for classification. Proteases, amylases and cellulases were studied through zymograms. In addition, a new antimicrobial zymogram was developed for a rapid identification of potential bacteriolytic proteins. The expression of several enzymes during encystment was also analysed using the mRNAseq data. One bacteriolytic protease and two encysting mediating proteases were identified.

8.2 Materials and Methods

A diagram of the materials and methods used is shown in Figure 49. A more detailed explanation of the protocols and experiments performed can be found in Chapter 2.

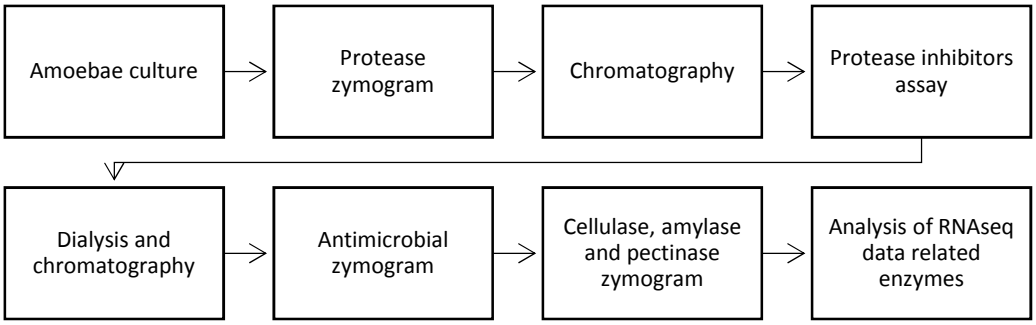


Figure 49. Diagram of methods used in Chapter 8 to characterise enzymes secreted by *Acanthamoeba* though zymograms.

8.3 Results

Zymograms were used to identify the secretions of *Acanthamoeba*. Protease zymograms were the focus of this chapter. The intention was to identify proteins involved in the digestion of bacteria. Once proteases were identified, an antimicrobial zymogram was developed to compare with protease zymogram results, identifying one protease capable of destroying bacteria. Finally, cellulase, pectinase and amylase zymograms were performed to build a more complete picture of *Acanthamoeba* secretions.

8.3.1 Protease zymograms and protease inhibitors

Several serine proteases from *A. castellanii* Neff strain were observed through gelatine zymograms. The proteases were identified as serine proteases as they were successfully inhibited with PMSF as seen in Figure 50. One of these proteases was identified to have a molecular weight close to 33 kDa. Using EDTA as a protease inhibitor substitute, slightly reduced the protease activity, but it did not inhibit the activity. Three different genotypes from two different morphogroups were tested in zymograms: T2 (morphogroup III), T5 (III) and T4 (II). Some genotypes showed different proteolytic patterns. However, there was no relation between morphogroups. Genotypes T4 and T5 (morphogroups II and II respectively) showed the same proteolytic profile, while T2 showed a different one. The proteolytic profile of five strains can be seen in Figure 51.

Other amoebae were tested for the secretion of proteases. Such amoebae were *Allovahlkampfia minuta* and *Vannella spp*. from strains KC and Arn. The tested *Vannella spp.* did not show any proteolytic activity in the supernatant. However, *A. minuta* did show slight activity during trophozoite stage. The results of the zymograms are shown in Figure 52 and Figure 53.

To try to modulate protease expression, samples were treated with pentoxifylline, propranolol and isoprenaline. There were no significant changes in protease expression as seen in Figure 54

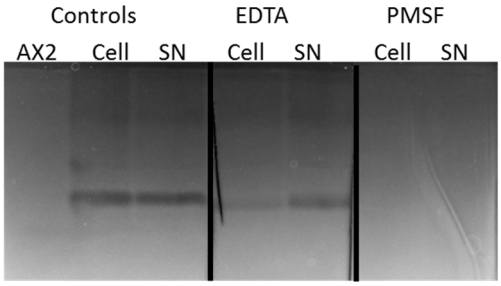


Figure 50. Protease inhibition zymogram. Protease zymogram using AX2 media as a negative control. All samples were from Neff strain and were treated with EDTA and PMSF as shown in the top of the gel. Two samples were loaded for each treatment: lysed cells and supernatant. The image was transformed into greyscale, colours inverted, and contrast altered to obtain a higher resolution of the bands.

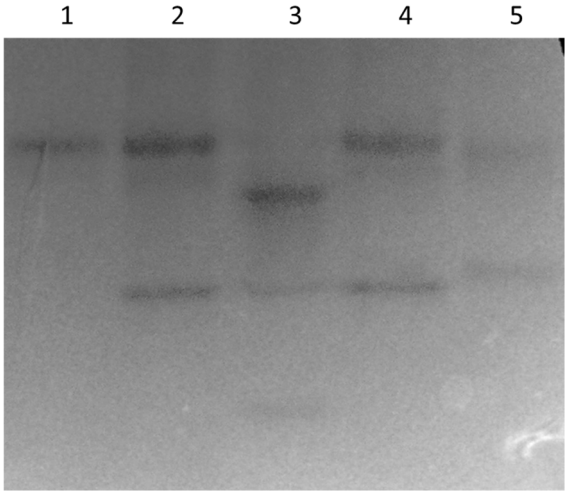


Figure 51**. Protease zymogram for five different *Acanthamoeba* strains. 1) Neff strain 2) Strain 65 genotype T4 3) Strain 61 genotype T2 4) Strain 64 genotype T4 5) Strain BC genotype T5. The image was transformed into greyscale, colours inverted, and contrast altered to obtain a higher resolution of the bands.**

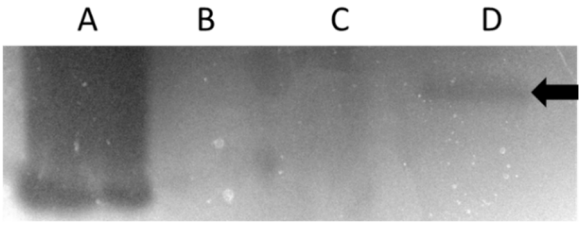


Figure 52. Protease zymogram from *Allovahlkampfia minuta*. A) AX2 supernatant from *Acanthamoeba* as a positive control. B) NS as a negative control. C) NS supernatant from *E. coli.* D) NS supernatant from *A. minuta*. The arrow points at the band formed by *A. minuta* proteases. The image was transformed into greyscale, colours inverted, and contrast altered to obtain a higher resolution of the bands.

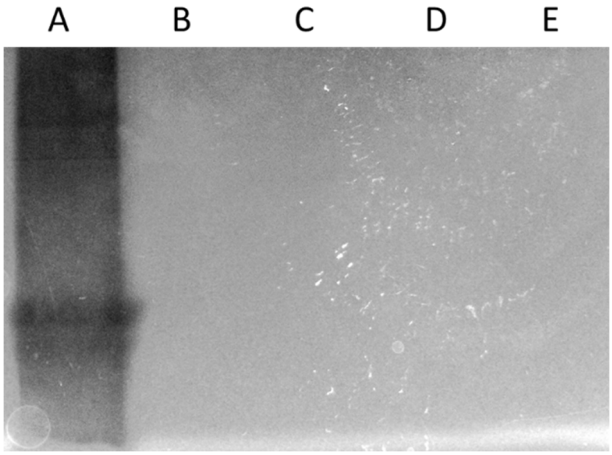


Figure 53. Protease zymogram from *Vannella spp.* A) AX2 supernatant from *Acanthamoeba* as a positive control. B) NS supernatant from *E. coli.* C) NS supernatant from *A. minuta* cysts. D) NS supernatant from *Vannella sp.* KC strain. E) NS supernatant from *Vannella sp.* Arn strain. The image was transformed into greyscale, colours inverted, and contrast altered to obtain a higher resolution of the bands.

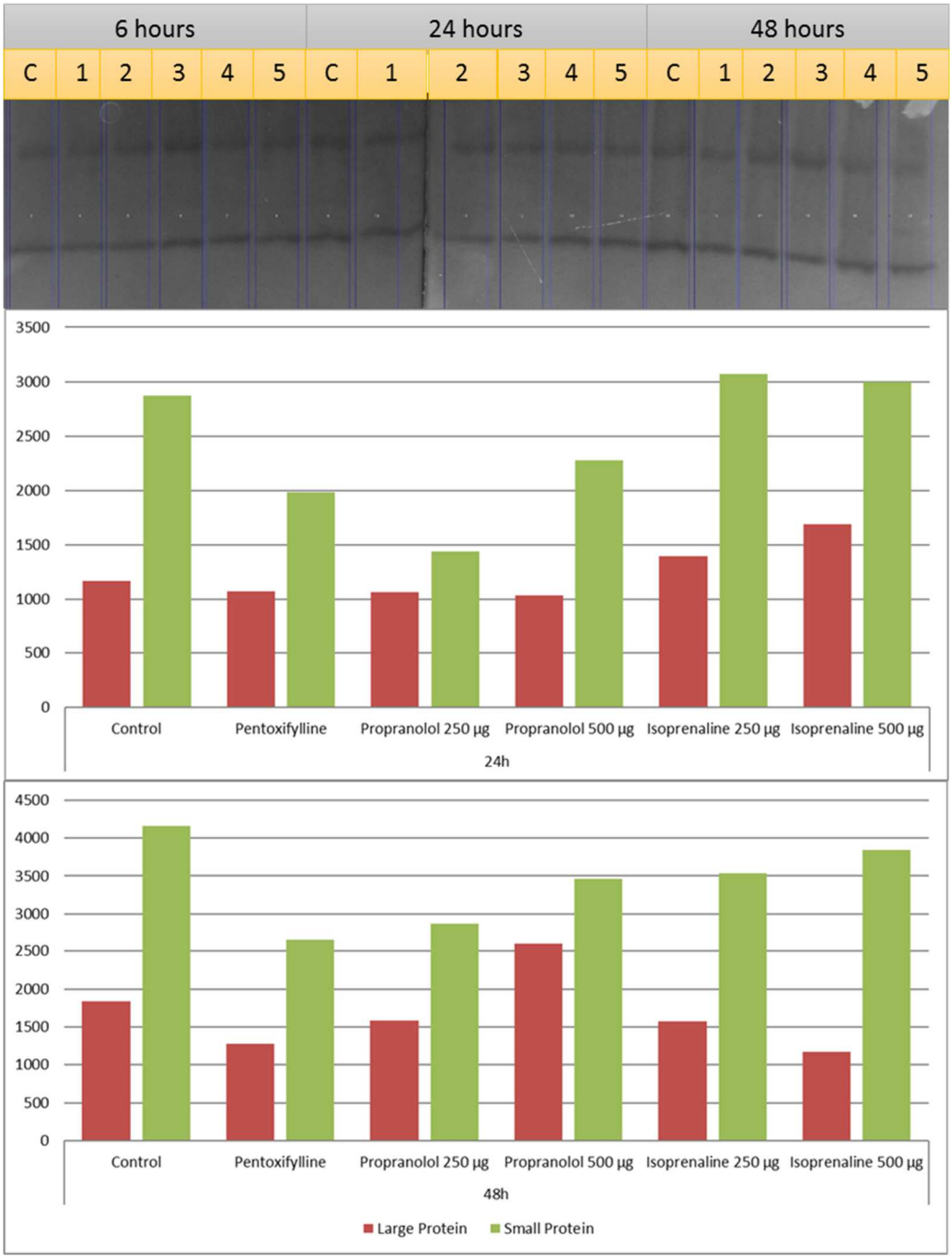


Figure 54. Protease effect of pentoxifylline (1), propranolol 250 µg (2), propranolol 500 µg (3), isoprenaline 250 µg (4) and isoprenaline 500 µg (5). At the top, protease zymogram with the eighteen samples that included the control and the five treatments after 6, 24 and 48 hours, from left to right respectively. The lanes were delimited, and the image was transformed into greyscale, colours inverted, contrast altered to obtain a higher resolution of the bands. The graphs show the number of pixels in each band (Measurement unit in the y-axis) under the curve estimated using ImageJ. The graph for 6 hours is not shown.

8.3.2 Chromatography for isolation of proteases

The secreted proteases from *Acanthamoeba* were not successfully isolated by chromatography. Isolating the protein required the repetition of several steps that include precipitation, dialysis and chromatography. By the time the steps had been performed, most of the proteins had been degraded. Partial results from chromatography are shown in Figure 55.

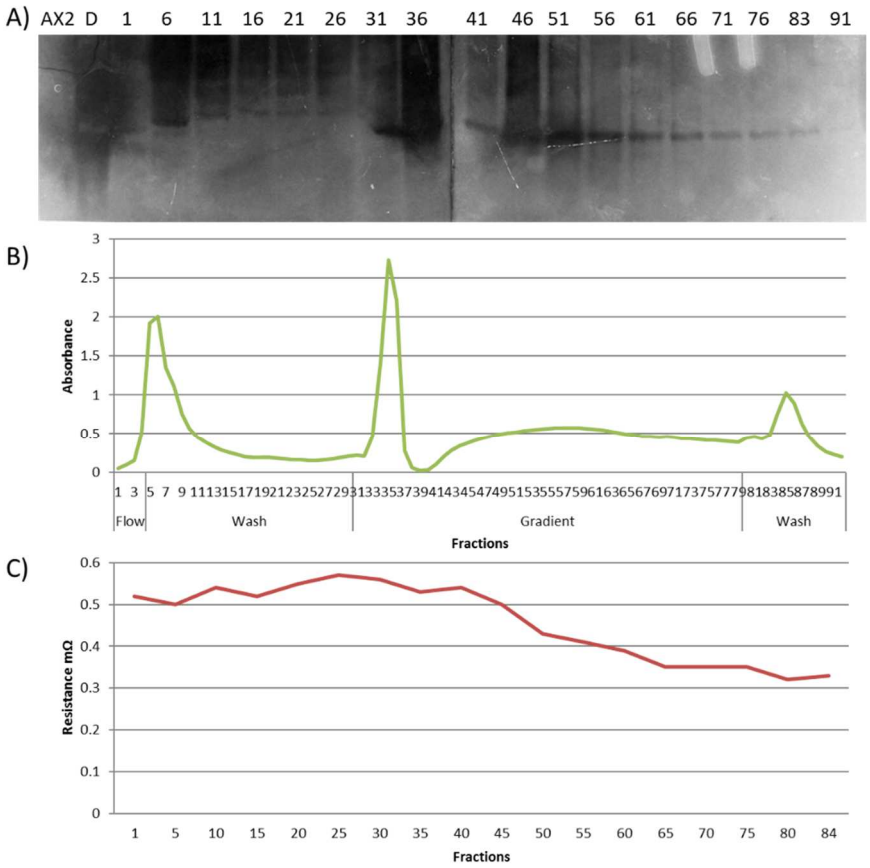


Figure 55. Chromatography results from protein precipitate from Neff strain. A) Protease zymogram where AX2 was used as a negative control, D shows the dialysate, and every number represents the number of the fraction loaded in the gel (7 µl each sample). The image was transformed into greyscale, colours inverted, and contrast altered to obtain a higher resolution of the bands. B) Absorbance read at 280 nm for all the fractions. 1-4 are the flow through after loading the sample. 5-30 are the fractions from the washing steps. 31 to 80 are the samples treated with a gradient of KCl. 80 to 91 are the samples after a final washing step. C) The resistance of selected samples was measured.

8.3.3 Development of an antimicrobial zymogram

One focus of the research was to identify potential antimicrobial proteins. Therefore, an antimicrobial zymogram was developed to observe bacteriolytic activity from protein extracts. The antimicrobial zymogram was tested with two different gram-negative bacteria *E. coli* and *Arcobacter butzlerii*. Both bacterial species were adequate for the antimicrobial zymogram.

Four different stains were tested for the antimicrobial zymogram. Such stains and their conditions are shown:

- Acridine orange (AO): Incubation for 20 min with 20 ml of H_20 with 0.02% of AO. Destain with H_2O.
- Coomassie: Incubating for 1 h with Coomassie stain. Destain with a destaining solution made of H_2O, methanol (10%) and acetic acid (5%).
- Methylene blue (MB): Incubation for 90 min with 20 ml of H_2O with 0.02 g of MB, 0.002 g NaOH and 0.002 g KCl. Destain with H_2O.
- Alcian Blue (AB): Incubation for 3 h in 18 ml of H_20, 2 ml of 95% ethanol and 0.06 g AB. Destain with H_2O.

Of the four stains, only Coomassie was able to resolve bands where antimicrobial activity happened. Both AO and MB did not destain. AB did not stain the gel. Results from the different stains are shown in Figure 56. Although, not evident in the Figure 56A, Coomassie stain did show a slight band of bacteriolytic activity. The ability of Coomassie to resolve bacteriolytic activity can be seen clearly in Figure 56B.

From the development of the antimicrobial zymogram, one protease was identified that had antimicrobial properties. This was verified as the incubation of the samples with PMSF inhibited the bacteriolytic activity (Figure 59). Incubation with EDTA lowered the bacteriolytic activity, but it did not inhibit it altogether, as it occurred in protease zymograms. This protease had an apparent size close to 33 kDa and was denominated Protease 33.

Different temperatures were also tested as seen in Figure 57. The antimicrobial protease was able to maintain bacteriolytic activity at high (50˚C) and low

temperatures (4˚C). Results were similar when using *A. butzlerii* as a substrate as seen in Figure 58. However, the highest temperature tested was 37˚.

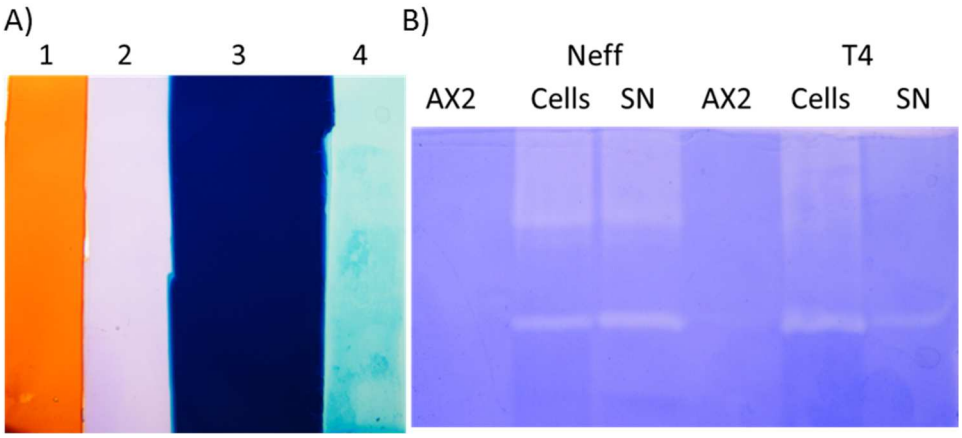


Figure 56. Comparison of different conditions for antimicrobial zymogram. A) Different stains were tested: 1) Acridine orange, 2) Coomassie blue, 3) Methylene Blue, 4) Alcian Blue. The pictured was cut to show the midsection where the bacteriolytic activity should have happened. B) Two different strains (Neff and strain 53) were run with cells' extract and supernatant (SN) and stained with Coomassie using AX2 media as a control.

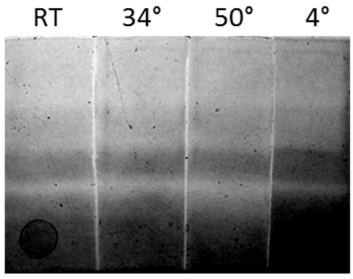


Figure 57. Temperature effect on antimicrobial zymogram. Four different temperatures were tested to see antimicrobial activity in a zymogram with *E. coli* as a substrate. Images were transformed into greyscale, and contrast altered to obtain a higher resolution of the bands.

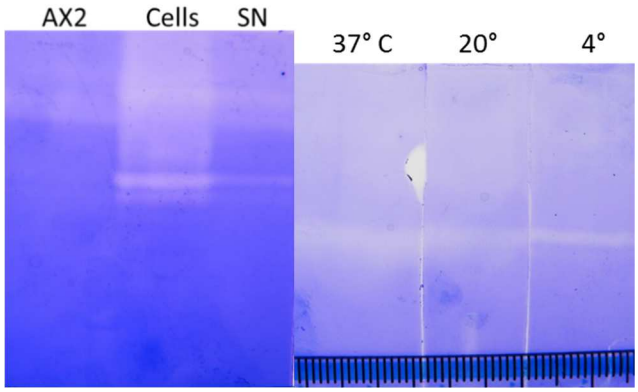


Figure 58. Antimicrobial zymogram with *A. butzlerii* as substrate. Left) The gel shows the antimicrobial zymogram substituting *E. coli* with *A. butzlerii* with loaded cells and supernatant (SN). AX2 media was used as a negative control. Right) Different incubation temperatures for antimicrobial zymogram using *A. butzlerii* as substrate and Neff strain supernatant as a sample.

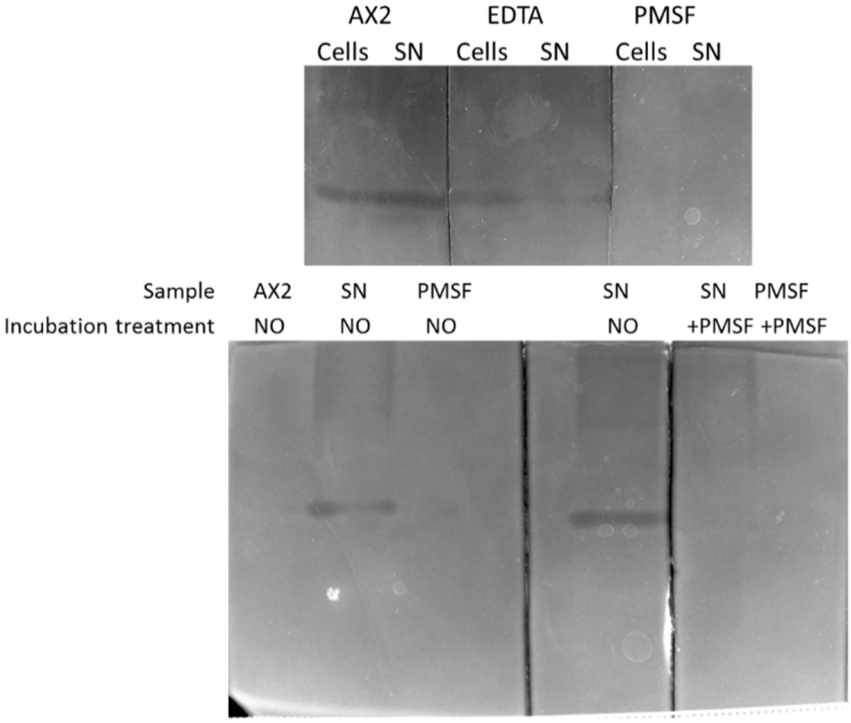


Figure 59. Antimicrobial zymograms with protease inhibitors. On top, antimicrobial zymogram with samples belonging to Neff strain. The gels were loaded with lysate cells and supernatant (SN). Samples were treated with EDTA and PMSF. On the bottom, antimicrobial zymogram with AX2 media as a negative control. All samples were from the supernatant (SN), but some were treated with protease inhibitor (PMSF). The "Incubation treatment" label refers to PMSF being added to incubation buffer after electrophoresis. Images were transformed into greyscale, colours inverted, and contrast altered to obtain a higher resolution of the bands.

8.3.4 Amylase, cellulase and pectinase zymograms

Additionally, zymograms were performed to test for amylase, pectinase and cellulase secretions from *Acanthamoeba* trophozoites. Cellulase and pectinase activity was not detected through zymograms. However, there was amylase activity from the supernatant. Results for amylase and cellulase activity can be found in Figure 60. Pectinase results are not shown.

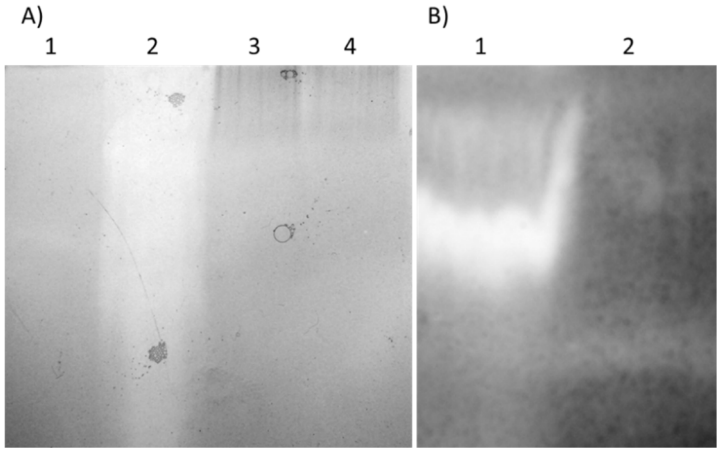


Figure 60. Cellulase and amylase zymograms. A) Cellulase zymogram with 1) Loading buffer as a negative control, 2) Cellulase (5 µl) as a positive control, 3) SN from Neff strain culture, and 4) Cell lysate from Neff strain. B) Amylase zymogram including 1) Amylase (15 µl) as a positive control and 2) Neff strain SN. Images were transformed into greyscale, and contrast altered to obtain a higher resolution of the bands.

8.3.5 Differential expression of enzymes during encystment through mRNAseq

Finally, the results from the mRNAseq from Chapter 7 were analysed to identify the expression profile of enzymes during encystation. AmoebaDB was used to search for records related to cellulases, lipases, amylases and proteases. The records found were used to identify enzymes involved in the encystation process with the RNAseq results. The results from all the records found and their expression during encystment (comparing 0 and 24 h only) are found in Appendix 7.

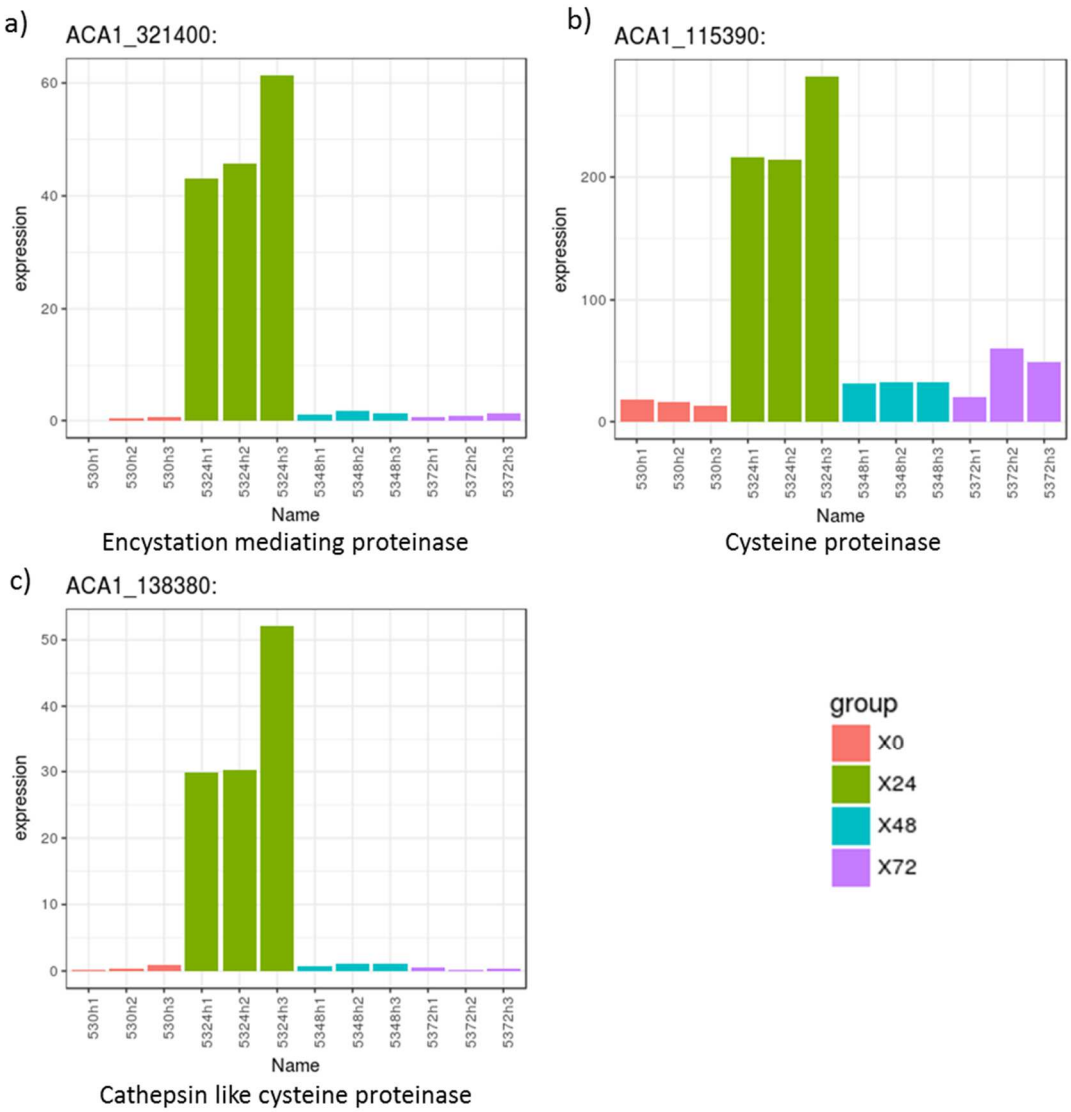


Figure 61. Expression profile of three encysting mediating proteases. The encystation mediating proteinase was used as a control as it has already been identified previously. The other two bar charts present similar profiles from two cysteine proteases. Each bar represents one sample of the four treatments: 0 hours (trophozoites), 24, 48 and 72 hours after addition of encysting media.

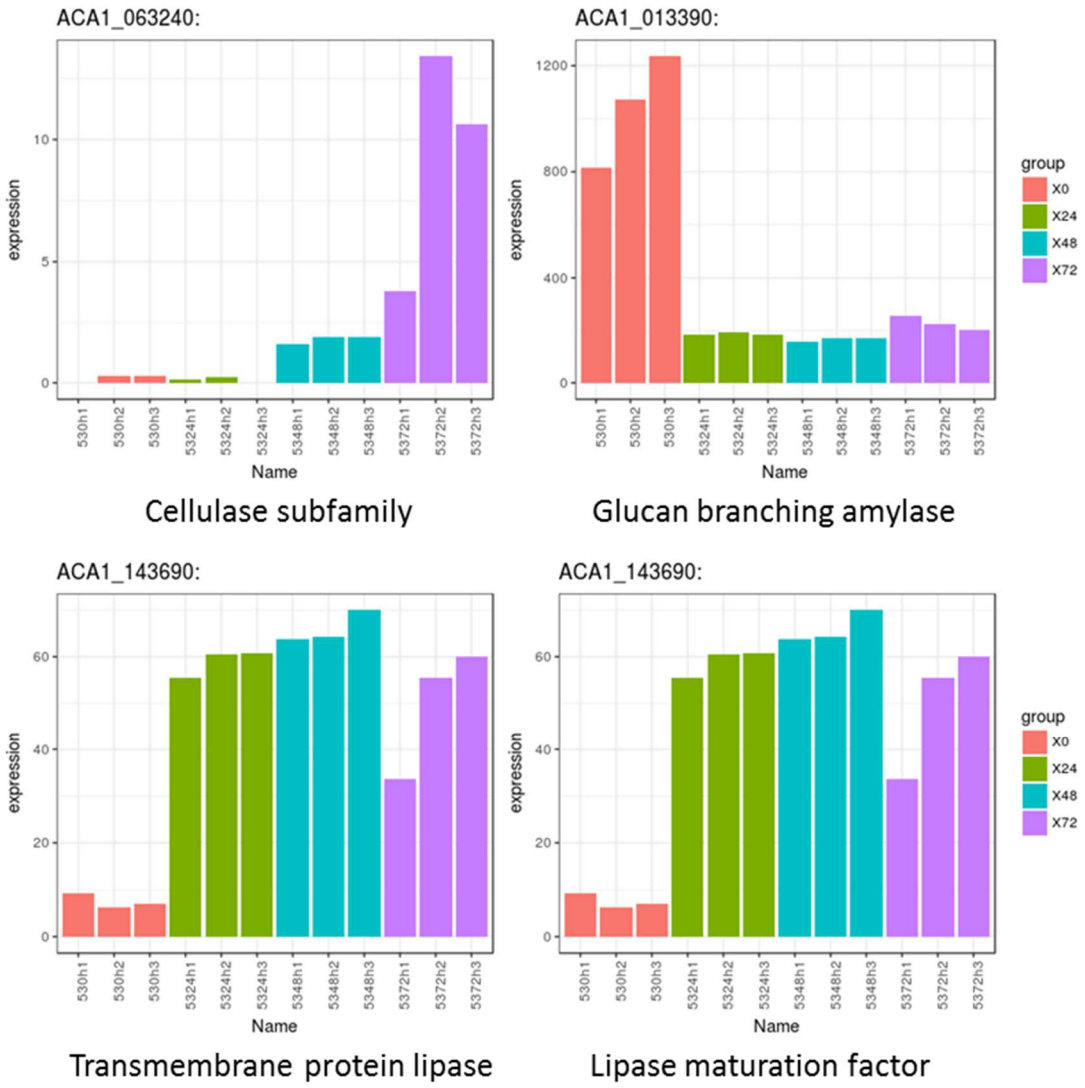


Figure 62. Differential expression during encystment of four different enzymes. Each bar represents one sample of the four treatments: 0 hours (trophozoites), 24, 48 and 72 hours after addition of encysting media.

Two new cysteine proteases involved in the encystation process were identified (Gene_IDs ACA1_115390 and ACA1_138380). Several other proteases were identified in the databases but were either just fragments of another protein or too similar to previously identified proteases. The expression profile of these proteases is found in Figure 61.

Other enzymes were identified that presented differential expression during encystment. The most relevant included a member of the cellulase subfamily (ACA1_063240), a glucan branching amylase (ACA1_013390), a transmembrane protein lipase (ACA1_143690) and a lipase maturation factor (ACA1_143690). Of

these enzymes, only the amylase was downregulated during encystment. The expression profiles can be found in Figure 62.

8.4 Discussion

Interactions between amoebae and bacteria play an essential role in the ecosystem. The interaction between *Acanthamoeba* and bacteria can result in bacterial lysis, a symbiotic relation or even amoebal lysis (Khan & Siddiqui, 2014). Like many other microbes, *Acanthamoeba* has developed protein secretions capable of killing or inhibiting the growth of bacteria (Iqbal et al., 2014). Some of those bactericidal factors are proteases, which standard eukaryotic proteases are not capable of doing.

Proteases are vital for *Acanthamoeba* as they serve several physiological purposes necessary for the survival of the microorganisms. One of such purposes is nutrition (Lorenzo-Morales et al., 2013). *Acanthamoeba* invasion is possible through the production of proteases that help them digest the invaded tissue. However, some proteases from *Acanthamoeba* do not produce tissue destruction, but tissue disruption where proteases alter cell junctions and produce separation (Omaña-Molina et al., 2013). Proteases can also increase cell permeability (Alsam et al., 2005b). Serine proteases of 130 and 150 kDa did not show cytotoxic effects but were capable of disrupting monolayers (Sissons et al., 2006). Other proteases, including a 42 kDa serine protease, are capable of degrading human corneal extracellular matrix (Cho et al., 2000; Piña-Vázquez et al., 2012). Furthermore, some of the serine proteases have been characterized as elastases (Ferreira et al., 2009). Some of the proteases produced by *Acanthamoeba* can be activated by contact. *Acanthamoeba* trophozoites secrete a mannose-induced serine protease with a weight over 100 kDa (Leher et al., 1998). However, *Acanthamoeba* secretes plasminogen activator by contact independent mechanisms (Mitra et al., 1995). Having so many different proteases, *Acanthamoeba* has shown to be able to produce proteases with several purposes.

Acanthamoeba produces three kinds of proteases: serine proteases, metalloproteases (Alsam et al., 2005b; Sissons et al., 2006) and cysteine proteases (Hadas & Mazur, 1993). Studies have shown that serine proteases and metalloproteases play an important role in pathogenicity (Cao et al., 1998; Hurt et al.,

2003). Since they play a role in infection, proteases might be an option for treatment, such as siRNA (Lorenzo-Morales et al., 2005b). The secretion of lytic proteins mediates invasion and tissue invasion. Therefore, the development of therapies focused on such proteases might help deal with infection (Piña-Vázquez et al., 2012).

Being capable of modulating protease production, could help deal with *Acanthamoeba*. G proteins are regulators of cellular activity related to proteolytic activity as they carry protease activated receptors capable of activating the innate immune response in humans through the production of antimicrobial peptides (Chung et al., 2004). Aqeel et al. discovered a similar relation between G proteins and protease secretion in *Acanthamoeba* (Aqeel et al., 2015). They showed that using inhibitors of β-adrenergic receptors such as propranolol could lower protease activity. However, a receptor agonist such as isoprenaline supposedly increased proteolytic activity. Results showed that the presence of propranolol did decrease slightly proteolytic activity (Aqeel et al., 2015). However, protease secretion did not increase in the presence of isoprenaline. The β-adrenergic receptors that are stimulated can also be increased with pentoxifylline. Mauduit et al. (1984) showed that lacrimal glands protein secretion could be altered using the three drugs. The results obtained did not show any significant change in protein secretion. Proteases experiments with isoprenaline, propranolol and pentoxifylline did not have a significant effect on protease production.

Cysteine proteases play a significant role in *Acanthamoeba* encystment (Leitsch et al., 2010). One cysteine protease has been identified as an encystment factor that plays an important role in autophagosomal mitochondrial degradation (Moon et al., 2012). At least two other proteases were identified in the current work that appear to be modulating encystment in *Acanthamoeba*. Unlike the previously reported serine protease, the newly identified proteases were recognised as papain-like cysteine proteases using Phyre2 protein modelling (Kelley et al., 2015). Future studies should look into silencing the encystation mediating proteases identified and the previously reported one (Moon et al., 2008b).

Another possible enzyme to target when dealing with *Acanthamoeba* are cellulases as one of the principal components of the cyst wall is cellulose (Blanton & Villemez, 1978; Tomlinson & Jones, 1962). *Acanthamoeba* has a cellulose degrading system

(Deichmann et al., 1977). The *A. castellanii* cellulase is similar to bacterial proteins, and it belongs to glycosyl hydrolase family 5 (Anderson et al., 2005).The study of the genome was able to identify several genes that code for cellulases (Clarke et al., 2013). At least one of those cellulases is overexpressed during encystment. Cellulases are important in other protozoans such as *Dictyostelium discoideum*, which produces a cellulase during spore germination (Blume & Ennis, 1991). The increase in cellulase expression during encystment can be attributed to the increase in cellulose in the organism.

Most living organisms need to be able to exploit polysaccharides found in the environment, but no animal type amylases are found in Amoebozoa organisms (Da Lage et al., 2007). It was shown in Figure 60 that *Acanthamoeba* trophozoites produce amylases that are downregulated during encystment, in accordance with the lower metabolic rate of cysts.

Other proteins involved in pathogenicity include adhesins such as the mannose-binding protein and laminin-binding protein (Garate et al., 2004; Hong et al., 2004). Furthermore, *Acanthamoeba* is capable of secreting ecto-ATPases of different molecular weights (Sissons et al., 2004). Also, glycosidases and phospholipases are secreted by *Acanthamoeba* and are factors in pathogenicity of the amoebae (Lorenzo-Morales et al., 2015; Matin & Jung, 2011; Mortazavi et al., 2011).

During encystment, several lipases are upregulated. In addition, a transmembrane protein (ACA1_143690) with the LMF1 superfamily domain that refers to transmembrane proteins that include lipase maturation factors. This process coincides with the degradation of molecules needed prior to the formation of the cyst (Leitsch et al., 2010).

Acanthamoeba secretions are vital in several processes, which include the two mainly studied processes during this study: nutrition and encystment. Understanding how *Acanthamoeba* is capable of lysing bacteria provides an opportunity to imitate such process to fight several bacterial infections. Understanding these processes might also help deal with *Acanthamoeba* as altering its feeding processes could prove of therapeutic importance.

Chapter 9 Cloning of antimicrobial proteins

9.1 Introduction

Based on the results from previous chapters, two potential antimicrobial proteins were selected for cloning: a lysozyme and Protease 33.

9.1.1 Protease 33

Proteases are hydrolytic enzymes of physiological and industrial importance (Rao 1998). Protease inhibitors and polyclonal antibodies were successful inhibiting *Acanthamoeba* destruction of corneal epithelial cells (Kim et al., 2006). A 33 kDa serine protease plays an important role in pathogenicity and invasion of corneal tissue. This protease was identified in *A. castellanii*, *A. healyii* and *A. ludgunensis* (Kim et al., 2003; Kong et al., 2000). The cDNA encoding this protease was amplified and cloned for characterisation.

9.1.2 Lysozyme

Lysozymes are hydrolases also known as muramidase or N-acetylmuramide glucanhydrolase. Lysozymes hydrolase peptidoglycan that covers bacterial cell wall, producing cell lysis (Masschalck & Michiels, 2003). Lysozymes were first observed by Laschtschenko as the bacteriolytic activity of chicken egg white (Laschtschenko, 1909). However, Alexander Fleming first coined the term “lysozyme”, after identifying several substances with bacteriolytic properties (Fleming & Allison, 1922). Lysozymes are ubiquitous enzymes that can be found in animals, fungi, plants and bacteria (Jollès & Jollès, 1984). A lysozyme from *Acanthamoeba* was first reported by Drozansky in 1968 (Drozański, 1969).

9.1.3 Chapter objectives

The objective was to clone Protease 33 and the lysozyme on *E. coli* for heterologous expression. The goal was to obtain purified proteins to be able to characterise their effect on different bacteria species. In the case of the protease, another objective was

to corroborate if the 33 kDa serine protease was Protease 33 shown to have antimicrobial properties.

9.2 Materials and Methods

A diagram of the materials and methods used is shown in Figure 63. A more detailed explanation of the protocols and experiments performed can be found in Chapter 2.

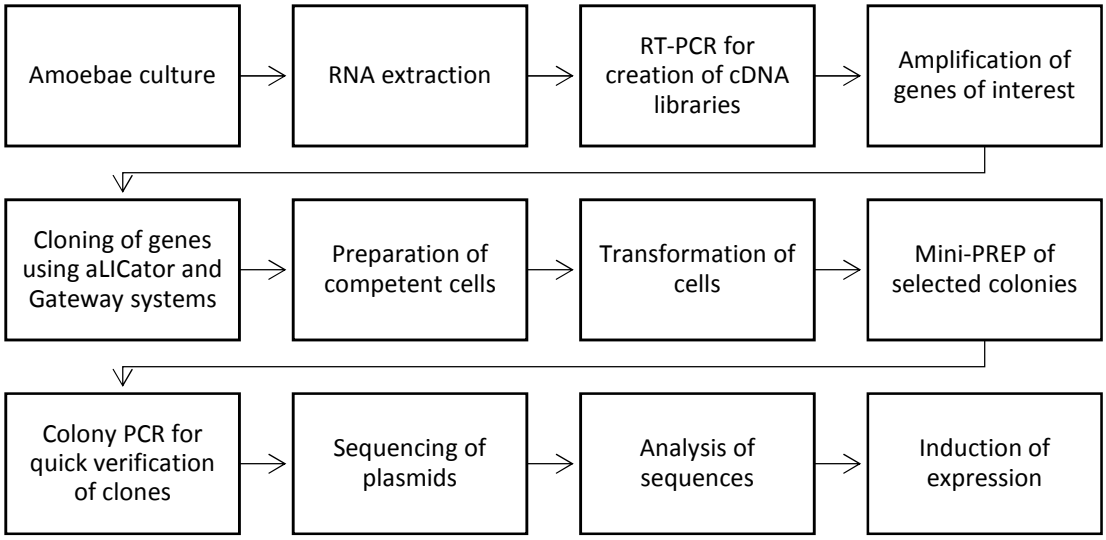


Figure 63. Diagram of methods used in Chapter 9 to clone selected proteins from *Acanthamoeba*.

9.3 Results

Protease 33 was identified through zymograms (protease and antimicrobial). In chapter 8, the protease with antimicrobial properties was estimated to have a size of 33 kDa. Through AmoebaDB and literature research, a similarly sized serine protease of 33 kDa was identified and selected for cloning. Additionally, literature research was used to look for other antimicrobial proteins. From this search, a lysozyme was selected. The sequence for the lysozyme was found through AmoebaDB and selected for cloning.

Once identified, primers for each gene were designed considering the different cloning systems. First, RNA was purified to form cDNA libraries. Then, both genes were successfully amplified from the cDNA libraries. Once amplified, the genes were cloned into expression vectors from the Gateway and aLICator cloning systems.

Plasmid maps for all the constructs can be observed in Chapter 2. The primers used for cloning are found in Appendix 2.

Heterologous expression of the proteins in *E. coli* was not possible with either cloning system. Different media (LB, 2xTY and TB), volumes, incubation temperatures and IPTG concentrations were tested to induce expression with negative results. Also, after cloning in BL21 DE3 *E. coli* strain did not work, a Rosetta 2 (DE3) pLysS genotype was tested with equally unsuccessful results. The Rosetta 2 (DE3) pLysS strain is specially designed to express toxic proteins and to translate codons that are rarely used in *E. coli.*

The translation of the obtained sequences aligned with the closest match through BLAST analysis are shown in Figure 64.

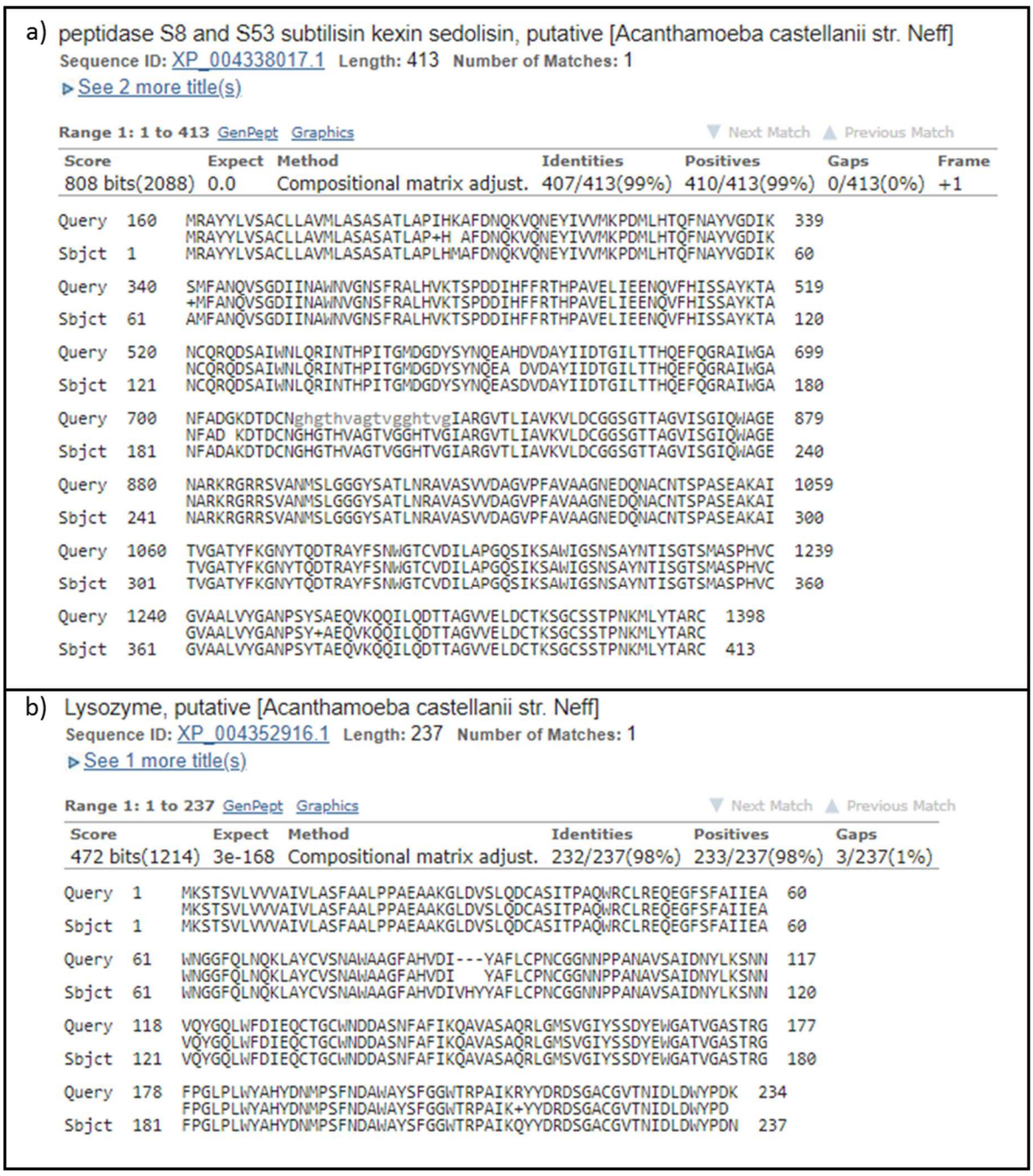


Figure 64. Protease and lysozyme full protein sequence compared with database entries. The images were obtained copying directly from NCBI BLAST webpage. The Query line shows the sequenced sample, the Sbjct the database entry.

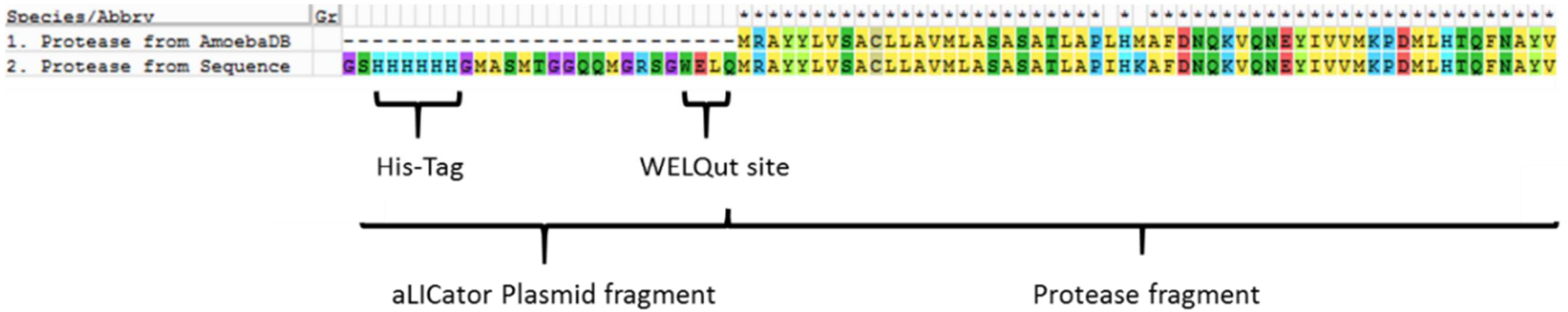


Figure 65. Translated fragment of Protease 33 as sequenced from pLATE52 from aLICator cloning system. The top sequence was obtained from AmoebaDB. The bottom sequence was obtained through sequencing of the plasmid. Image cropped from MEGA 7.

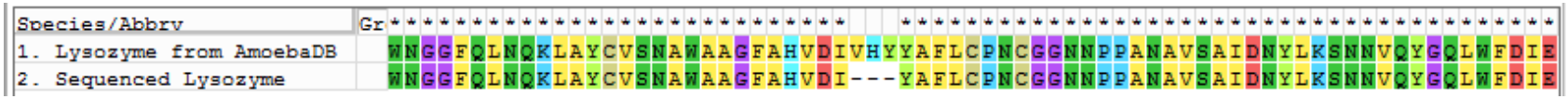


Figure 66. Comparison fragment of the amino acid sequences for lysozyme from database and sequencing. Shows the fragment where three amino acids are missing. Image cropped from MEGA 7.

Species/Abbrv	Group Name	*
1. Lysozyme from amoebaDB		GFAHVDIVHYYAFLCPNC
2. Sequenced Lysozyme		GFAHVDI---YAFLCPNC
3. XP 020428293.1 glycoside hydrolase family 25 protein Polysphondylium pallidum PN500		GFQTVDL---YAFLCNQC
4. XP 644554.1 component of the counting factor complex Dictyostelium discoideum AX4		GFDNIDL---YAFLCSEC
5. XP 004359505.1 glycoside hydrolase family 25 protein Dictyostelium fasciculatum		GLS-VDI---YAFLCNQC
6. KYR00416.1 glycoside hydrolase family 25 protein Dictyostelium lacteum		GFQHVDI---YMFLCNQC
7. XP 643144.1 glycoside hydrolase family 25 protein Dictyostelium discoideum AX4		GLS-VDL---YAFLCSQC
8. XP 004347089.1 glycosyl hydrolase Acanthamoeba castellanii str. Neff		GMS--HVDVYMFPCP---
9. KYR00374.1 glycoside hydrolase family 25 protein Dictyostelium lacteum		GMA--DVDIYVFPCF---
10. XP 004347091.1 glycosyl hydrolases family 25 subfamily protein Acanthamoeba castellanii st		GMS--HVDVYMFPCP---

Figure 67. VHY deletion alignment comparing 10 lysozyme sequences.

9.3.1 Cloning of protease 33

The gene assumed Protease 33 (ACA1_069730) was successfully amplified from cDNA obtained from *Acanthamoeba* Neff strain axenic cultures. The theoretical molecular weight from the sequence of the protease was 44 kDa. The plasmid was then sent for sequencing to Edinburgh Genomics. The sequence of the cloned protease gene showed several mutations. The mutations resulted in six amino acids changes compared to the sequence reported in AmoebaDB. However, the changes did not alter the reading frame. A fragment of the sequence including part of the plasmid can be observed in Figure 65.

Since the protease selected from *Acanthamoeba* was a secreted protein found in the supernatant, a hydropathy plot was created to look for hydrophobic domains that could indicate secretion from the cell. The Protease 33 sequence contains a positive hydrophobic domain in the N-terminal, indicating such possibility. The hydropathy plot can be observed in Figure 68.

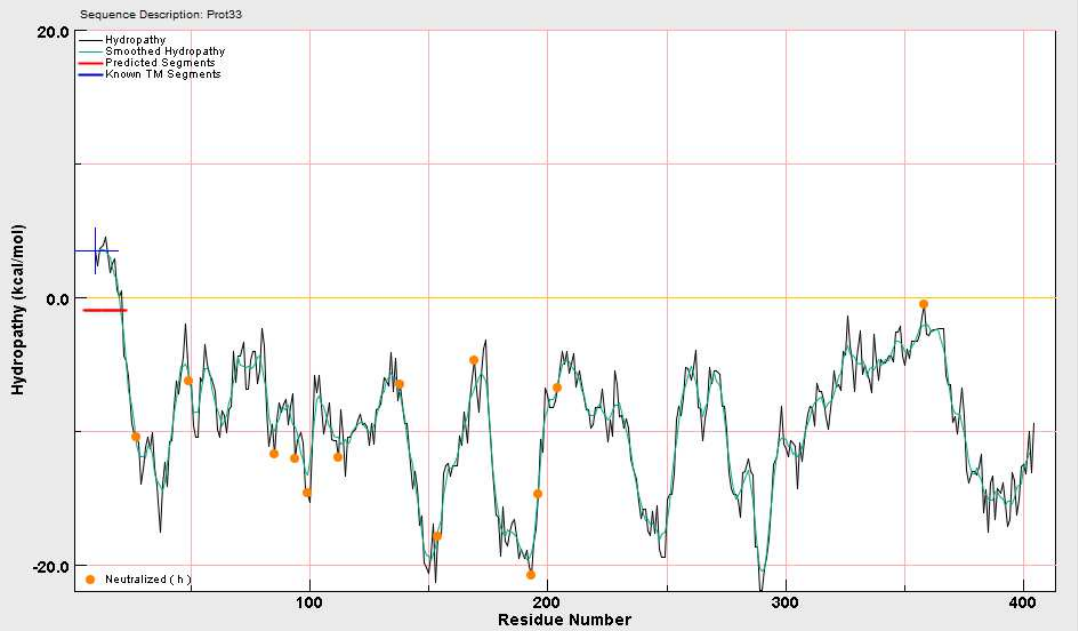


Figure 68. Hydropathy plot for Protease 33. The red line shows the hydrophobic domain at the N-terminal of the protein. The hydropathy plot was created using MPEx software.

.

9.3.2 Cloning of the lysozyme

The lysozyme gene (gene id ACA1_069730) could not be amplified from cDNA libraries created from RNA extractions obtained from *Acanthamoeba* Neff strain grown in axenic cultures. However, the gene was successfully amplified when the RNA extraction was performed from co-cultures of *Acanthamoeba* Neff strain and *E. coli*. Once isolated, cDNA libraries were used to clone the gene into the appropriate plasmids. The plasmids were sent for sequencing to verify the sequence of the gene through comparison with the database sequence. Both sequences had high similarity but were not exact. Once translated, the cloned sequence presented the deletion of three amino acids in the 89 to 91 positions of the protein as seen in Figure 66. These three amino acid were valine, histidine and tyrosine (VHY) in the original sequence. Another mutation resulted in the change of one glutamine for an arginine. However, these mutations did not alter the reading frame of the protein.

To identify lysozymes with a similar VHY deletion, a BLASTp was performed with the sequence obtained. From the top 100 results, 49 were selected that included the words “lysozyme” or “glycoside hydrolase.” The 49 records were aligned with the sequences from the database and our sequenced result. There were four other species that presented the same deletion: *Polysphondylium pallidum, Dictiostelyum discoideum, Dictiostelyum fasciculatum* and *Dictyostelium lacteum.* All four species are slime moulds belonging to the Dictiostellidae family. The fragments showing the VHY deletion in the different species are shown in Figure 67.

Analysis of the cloned sequence showed a protein with a molecular weight of 25.8 kDa. The lysozyme also contains a positive hydrophobic domain in the N-terminal indicating the possibility of secretion from the cell. The hydropathy plot can be observed in Figure 69.

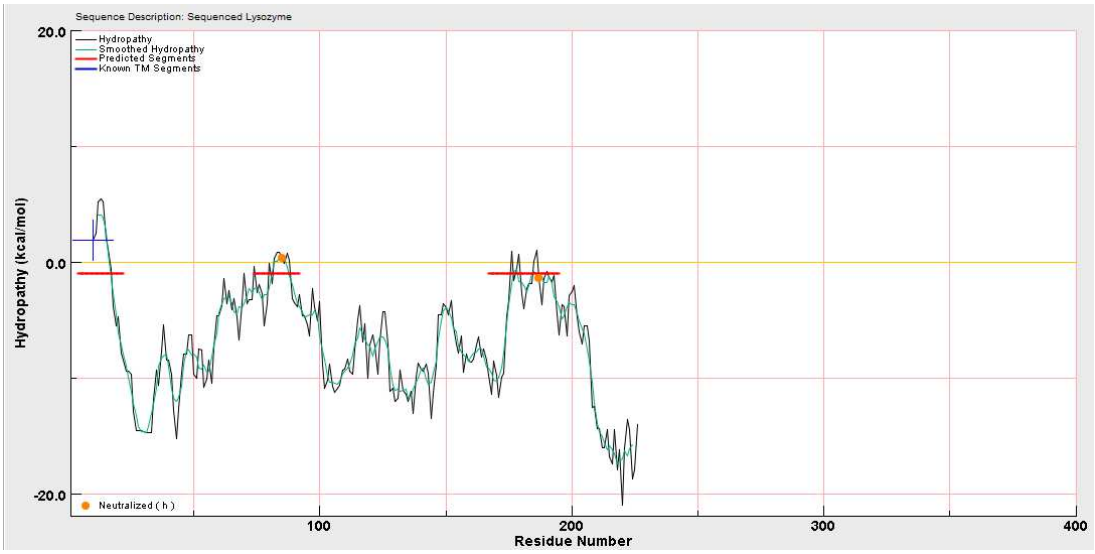


Figure 69. Hydropathy plot for *Acanthamoeba* lysozyme. The red line shows the hydrophobic domain at the N-terminal of the protein. The hydropathy plot was created using MPEx software.

9.4 Discussion

Production of lytic proteins by protozoans is a common defence mechanism against other microorganisms. One example is the hydrolysis of antimicrobial peptides by a protease expressed by *Leishmania* (Kulkarni et al., 2006). Proteases can also activate the production of antimicrobial peptides and modify precursors leading to the formation of such peptides (Faye et al., 2004; Lee et al., 2010; Shinnar et al., 2003). Some eukaryotes have proteins capable of developing antimicrobial properties when in contact with bacterial proteases (Corrêa et al., 2011; Tagai et al., 2011). Moreover, protease inhibitors play a role in inflammation, extracellular matrix synthesis and tissue repair (Hiemstra, 2002). Protease inhibitors can have bactericidal effects. Two examples are the human secretory leukocyte protease inhibitors and protease inhibitors isolated from frog skin secretions (Ali et al., 2002; Doumas & Kolokotronis, 2005). Since Protease 33 is secreted in axenic cultures, it might have a role in prevention and defence. However, the lysozyme was only expressed when in the presence of prey, indicating a role in digestion more than defence.

Proteases capable of bacterial lysis, such as Protease 33, have been researched for some time. The most common examples of such proteases come from prokaryotes. One such example is lysostaphin, which is an antimicrobial protease produced by

Staphylococcus simulans against *Staphylococcus aureus* (Schindler & Schuhardt, 1964). However, the potential of prokaryotic proteases against other prokaryotes is limited as gene resistance could spread rapidly through horizontal gene transfer (Bardelang et al., 2009). Nonetheless, eukaryotic proteases with potential bactericidal capabilities are another option as they are less likely to be affected by gene transfer and produce antimicrobial resistance. Unfortunately, eukaryotic proteases with bactericidal capabilities are scarce. An aspartic protease with antimicrobial properties is found in potatoes (Guevara et al., 2002). A 43 kDa serine protease with activity against gram-negative bacteria was isolated from horseshoe crabs (Kawabata et al., 1996). Additionally, a 48 kDa serine protease was isolated from jackfruit (*Artocarpus heterophyllus*) latex (Siritapetawee et al., 2012). Proteinase 3 from neutrophils has also been identified as a serine protease with potential antimicrobial activity (Campanelli et al., 1990). Protease 33 has also shown bactericidal capabilities. However, we were not able to confirm that the 33 kDa protease reported in the literature was the Protease 33 observed in the antimicrobial zymograms (Hong et al., 2000; Kim et al., 2003, 2006). Successful expression and characterisation of the protease are needed to identify the protease efficiently and to verify the sequence. New chromatography techniques could help isolate the protein from the supernatant in a faster manner allowing for direct characterisation.

Drozansky (1971; 1969)first identified the lysozyme from *Acanthamoeba* and found that a pH of 5.0 was optimal for its function. Vacuoles of some protozoans become acidic soon after ingesting food (Drozanski, 1969; Greenwood & Saunders, 1891; Howland, 1928; Le Dantec, 1891). The presence of lysozyme can help prevent antimicrobial growth within digestive vacuoles helping in nutrition (Leippe, 1999). Korn and Olivecrona (1971) also identified a membrane protein in *Acanthamoeba* with similar characteristics to a lysozyme. The study of *Acanthamoeba'*s genome identified at least one putative lysozyme that could depolymerise bacterial peptidoglycan (Anderson et al., 2005; Clarke et al., 2013). Other protozoans such as *Dictyostelium dyscoideum*, *Entamoeba histolytica* and another unidentified free-living amoeba produce lysozymes to degrade bacterial cell walls (Berger & Mannheim, 1970; Glöckner et al., 2002; Jacobs & Leippe, 1995). The putative lysozyme found in the database was not expressed when the amoebae were grown axenically. However, the lysozyme is expressed when *Acanthamoeba* is grown in co-culture with *E. coli*. The need of bacteria for the expression of the lysozyme indicates a role in bacterial

lysis. There was no significant change in lysozyme expression during encystment (Appendix 7).

The lysozyme being produced by *Acanthamoeba* Neff strain lacks the three amino acid sequence VHY. This sequence was also lacking in lysozyme records for three different species of the genus *Dictyostelium* and one from *Polysphondylium,* indicating that the mutation should not inhibit the function of the protein*. Dictyostelium* and *Acanthamoeba* have been evolutionarily linked to macrophages (Cosson & Lima, 2014; Siddiqui & Khan, 2012a). Bacterial lysis is essential for *Acanthamoeba, Dictyostelium dyscoideum* and macrophages. This presents more evidence to the possible evolutionary relation between amoebozoan organisms and macrophages.

The heterologous expression of the proteins was not achieved even though the genetic sequences proved to be satisfactory. Both sequences showed mutations, however none of them altered the reading frame or translated a stop codon that would impede the translation of the protein. Logically, the production of bactericidal proteins in *E. coli* is challenging as they interfere with homeostasis of the host cell (Rosano & Ceccarelli, 2014). To try to produce the proteins despite this challenge, we used the aLICator system and strains carrying the pLysS genotype that present a tight control of expression and have tuneable expression systems. These options are recommended for the production of toxic proteins. However, none of the options used was successful. The use of IPTG to induce expression could be modified in future experiments since dose-dependent induction is not possible with IPTG as it enters the cell through active transport (Fernández-Castané et al., 2012). New inducers or tuneable promoters should be tested. The hydropathicity of both proteins shows the hydrophobic domains in the N-terminal characteristic of extracellular proteins (Baneyx, 1999). Future alternatives for production might include using yeast, insect cells or cell-free systems. Other options might come from the myriad of novel technologies that are continuously being developed. One such option is encapsulins that can use as nano-compartments in the production of cytotoxic molecules (Giessen & Silver, 2016).

Besides Protease 33 and the lysozyme, other potential antimicrobial protein to target could be pore-forming proteins. Pore-forming proteins are well-known factors of pathogenicity in bacteria and protozoans. Such proteins are considered essential for

the destruction of the host tissue in parasites. Michalek et al. (2013) discovered one such protein from pathogenic *A. healyi* called acanthaporin. Acanthaporin function is lysis of the nutrient source on which it is feeding (Leippe, 2014). Other amoebae like *Entamoeba hystolitica* and *Naegleria fowlerii* have this kind of pore-forming proteins (Herbst et al., 2002; Leippe et al., 1991). Primers developed to amplify acanthaporin did not work in *A. castellanii* isolate used here.

The secretion of lytic proteins mediates invasion of tissue and lysis of other microorganisms (Pina Vazquez 2012). Therefore the study of such proteins, whether proteases, lysozymes or any other, offer the opportunity to treat disease caused by either the organisms producing the enzyme or the organisms affected by it. The study of antimicrobial proteins from *Acanthamoeba* could help develop new therapies against bacteria and *Acanthamoeba* itself. Enzymes expressed as the amoebae excyst, for example, may be used to digest the cyst wall in the infected cornea. Understanding the pathogenic factors can help develop new alternatives.

Chapter 10 Conclusions

The importance of *Acanthamoeba* for human health has been steadily growing over the last 50 years. However, there is a lack of efficient drugs to treat the infection. Therefore understanding the pathogenic factors and encystation can help deal with the pathogen. This work consisted of three main aspects. First, the isolation and identification of new strains of *Acanthamoeba* and other amoebae. Second, the identification of encystment factors from *Acanthamoeba*. Finally, the identification and characterisation of antimicrobial proteins produced by *Acanthamoeba*.

Several strains of *Acanthamoeba* from different genotypes were isolated. Additionally, new strains of *Vannella* were isolated alongside new species *Allovahlkampfia minuta* and *Leptomyxa valladaresi*. A higher understanding of *Acanthamoeba* systematics might clarify some aspects of the pathogenesis and the ecology of the organism. Furthermore, discovering new amoebae might help identify new antimicrobial factors. Finally, identification of new amoeba species and strains can help understand microbial communities, further clarifying the relationship between bacteria and protozoans in the environment.

Second, understanding encystation can help decrease the number of relapses and increase the effectiveness of therapies against *Acanthamoeba* infection. The identification of encystation factor in this study can help deal with persistence in AK. Further characterisation of such encystation factors can help identify the critical aspects involved in differentiation. Future studies with siRNA or available drugs could be used to elucidate the mechanisms and pathways critical for encystment. Another important step towards the comprehension of encystment would be a higher understanding of the metabolic pathways of *Acanthamoeba*.

Researching protein secretions and pathogenic factors can help decrease infection and their effects. It might also help deal with some bacterial infections, as it is known that *Acanthamoeba* produces proteins with antimicrobial functions. Further studies and characterisation of Protease 33 and *Acanthamoeba* lysozyme are required. Alternative expression systems could be tested for the heterologous expression of the proteins.

Increase in the understanding of *Acanthamoeba* biology is essential to develop effective treatments against the different infections caused by the pathogen. Two of the keys towards dealing with such infections are the secreted proteins and the encystment factors. If we understood these aspects of *Acanthamoeba* biology, the virulence and persistence of these infections could be mitigated, and new treatments could be developed.

Appendix 1. Publications

Experimental Parasitology 183 (2017) 85–91

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Leptomyxa valladaresi n. sp. (Amoebozoa, Tubulinea, Leptomyxida), from Mount Teide, Tenerife, Spain

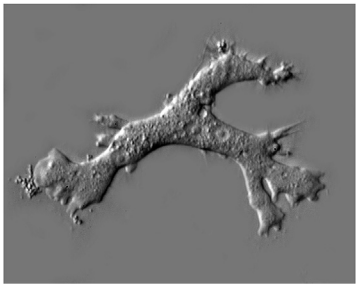
Alvaro De Obeso Fernandez Del Valle [a], Jacob Lorenzo-Morales [b], Sutherland K. Maciver [a, *]

[a] Centre for Integrative Physiology, Biomedical Sciences, Edinburgh Medical School, University of Edinburgh, Hugh Robson Building, George Square, Edinburgh, EH8 9XD, Scotland, UK
[b] University Institute of Tropical Diseases and Public Health of the Canary Islands, University of La Laguna, La Laguna, Tenerife, Canary Islands, Spain

HIGHLIGHTS

- A new species of *Leptomyxa* is described.
- Forms two different locomotory morphology one cylindrical the other flattened and fan like.
- Feeds on bacteria and *Acanthamoeba.*
- Contains endosymbiont bacteria which may produce geosmin.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 28 July 2017
Received in revised form
11 August 2017
Accepted 11 September 2017
Available online 16 October 2017

Keywords:
Amoebozoa
Leptomyxida
Systematics
Phylogeny

ABSTRACT

Leptomyxa valladaresi was isolated from soil in a pine forest on the southern flank of Mt Teide in Tenerife, Spain. It feeds on bacteria and on a range of other amoebae, and it was possible to establish bi-axenic cultures with *L. valladaresi* and *Acanthamoeba*. It is easily propagated on a *E. coli* also. 18S rDNA gene sequence analysis suggests that it is most closely related to *Leptomyxa variabilis*, however this amoeba differs in important detail. *L. valladaresi* is primarily mononucleate whereas *L. variabilis* is multinucleate. *L. valladaresi* is a larger amoeba and although the cysts are similar in size, there is no sign of the pore-like structures described in *L. variabilis* cysts. *L. valladaresi* can adopt a rapid monopodal and tubular morphology similar to that described for *L. neglecta* and *Rhizamoeba matisi*, and is never reticulated as larger *L. variabilis* individuals tend to be. The mean generation time was found to be 18 h, in line with amoebae of this size. Like other members of the genus, *L. valladaresi* is reported to harbour intracellular, presumably endosymbiotic bacteria, and a *Delftia* sp has been identified by 16S PCR a bacterium which is also known to grow within *Acanthamoeba*. The availability of this easily cultured species will help to characterize of this little studied genus and family and their relationship with bacteria, both prey and symbionts.

1. Introduction

Amoeboid organisms belonging to the order Leptomyxida have been isolated from a variety of environments including saltwater (Page, 1983; Kühn, 1996), freshwater (Smirnov et al., 2009), temperate soils (Brown & Smirnov, 2004), soils above the

* Corresponding author.
E-mail address: smaciver@ed.ac.uk (S.K. Maciver).

https://doi.org/10.1016/j.exppara.2017.09.017
0014-4894/

permafrost (Shmakova et al., 2013), and soils from deserts (Bamforth, 2008). Order Leptomyxida contains an unusual assortment of medium to very large amoebae some attaining a length of 3 mm in some cases, making them amongst the largest amoeboid organisms (Singh, 1948). This group display a variety of different morphologies, not only between species but individuals within the same species are often found to adopt very different morphologies and to be able to switch rapidly between them. Some members produce fan-shaped flattened trophozoites (similar to the Vannellae), some that produce adhesive root-like ramose processes, and others that produce simple tubular, limax forms which advance rapidly, led by a hemispherical hyaline cap. As a result of this heterogeneity in form and habit, many genera and species have been described and the historic state of the *Leptomyxida* was one of disarray (Smirnov et al., 2008, 2009).

A recent comprehensive phylogenetic and morphological analysis of the *Leptomyxida* has brought clarity (Smirnov et al., 2017). The order now been reorganised into three genera. Genus *Flabellula* (uniting the genus *Paraflabellula*, some members of genus *Rhizamoeba* and the pre-existing genus *Flabellula*), genus *Rhizamoeba* (presently consisting of one named species, *R. saxonica*, CCAP1570/2) and genus *Leptomyxa*. A small number of the genus *Leptomyxa* have been described (Table 2) and only two are currently available in the main culture collections. The strain described here is easy to culture and maintain, and its availability will hopefully help characterize this interesting group.

Free living amoebae (FLA) are known to produce infection in humans (Schuster and Visvesvara, 2004; Visvesvara et al., 2007), one of these, *Saccamoeba* is within the Tubulinea (Gelman et al., 2001; Walochnik et al., 2010. The heterolobosean, *Naegleria fowleri* causes a usually fatal meningitis (Carter, 1970), and while the T4 genotype of *Acanthamoeba* is the most abundantly encountered type it is also known to be disproportionately associated with human infection (Maciver et al., 2013), the most common of which is *Acanthamoeba* keratitis (Lorenzo-Morales et al., 2015). *Balamuthia mandrillaris* is a third major FLA which causes human fatalities (Maciver, 2007). This amoeba was first described as being a Leptomyxid amoebae because of its superficial resemblance to this group (Visvesvara et al., 1990), but it is presently clear that genus *Leptomyxa* and the groups immediately related to it, do not contain pathogens. Even if FLA species are not directly pathogenic many harbour intracellular bacteria that may be human pathogens (Mohamed et al., 2016). It is therefore important to understand FLA diversity and their interaction with bacteria (Walochnik et al., 1999).

2. Methods and materials

2.1. Cell culture

Acanthamoeba (Neff strain, ATTC30010, CCAP1501/1B) was cultured axenically in 75 ml flasks. *Vermamoeba vermiformis*, *Vannella pentlandii* (CCAP 1589/23) and an unidentified vahlkampfiid amoebae were cultured on YME plates (2% agar, 0.01% yeast extract, 0.025% malt extract) with *E. coli* as previously described (Maciver et al., 2018). The yeasts *Pichia pastoris* and *Rhodotorula mucilaginosa* were cultured on 1.5% agar plates with 0.01% malt extract and 0.01% yeast extract. *E. coli* (BL21 de3) was cultured on LB plates or grown in liquid LB media.

Table 1
Parameters of *L. valladaresi* cyst size and the frequency of nuclei number.

	Single nucleus	Double nuclei	Triple nuclei
Diameter (μm)	22.42	28.91	35.62
Frequency (%)	77%	16.4%	6.6%

2.2. Isolation of amoebae

A small sample of dry soil was taken from a dry gully in a large forested area consisting predominantly of the Canary Island Pine (*Pinus canariensis*) from a site known, (not very helpfully) as “Las Lajas” meaning “the Rocks” at lat.28.1886° long. −16.6644° and at an altitude of 2076 m on the southern flank of Mount Teide, Tenerife, Canary Islands, Spain. Amoebae were isolated by plating soil in Neff's saline plates overnight and then inverting small strips onto fresh non-nutrient agar plates layered with *E. coli*. After several days *Acanthamoeba* was found to grow out, and a much larger amoeba (strain LLT) was subsequently observed to grow out over the *Acanthamoeba* dominated regions of the plate, until very few *Acanthamoeba* could be found. *Acanthamoeba* were easily identified on the plates by the characteristic appearance of the cysts. LLT was initially cloned by excising small regions off agar plates and placed in micro wells containing trophozoites of *Acanthamoeba* (Neff strain). LLT was found to grow on a bacterium from the original source and was routinely cultured with this bacterium or *E. coli* (BL21 de3) on YME plates (see above).

For long term storage amoebae and cysts were washed in Neff's saline and then taken up in 12% DMSO in Neff's incubated at room temperature, then overnight at −20 °C then finally placed at −80 °C. Cultures were revived by thawing out to room temperature, washing with Neff's saline by low speed centrifugation, and either fed with *Acanthamoeba* in Neff's saline to a culture flask, or placed on *E. coli* spread YME plates. Additionally, LLT were stored at room temperature in clay pellets as previously described (Lorenzo-Morales and Maciver, 2006).

Light micrographs and measurements were performed on a Leica inverted microscope with Hoffman modulation contrast microscope and a laptop controlled Canon EOS digital camera. Additional micrographs were taken using a phase contrast microscope. Videos were recorded using “EOS camera movie record 0.3” (eos-movrec.sf.net). For the enumeration of nuclei, amoebae were fixed with Nissenbaum's fixative (Nissenbaum, 2001) and stained with Kernechtrot nuclear fast red (Fluka).

2.3. Genomic DNA purification and PCR

Genomic DNA was isolated and purified as previously described (Lorenzo-Morales et al., 2005; Maciver et al., 2018). The eukaryotic 18S rDNA gene was amplified using several primer pairs as previously described (Corsaro and Venditti, 2010). CAT1 (5′-CAT GCA TGT CTA AGT ATA AGC-3′) with GSPr (5′-TTC AC <G/A> GTA AAC <G/A> ATC TGG GC-3′), or 1137R (5′-GTG CCC TTC CGT TCA AT-3′), and 892 cF (5′-GTC AGA GGT GAA ATT CTT GG-3′) with Br (5′-GAT CCT TCT GCA GGT TCA C-3′). Bacterial 16S rDNA genes were amplified using universal prokaryote primers 16S Fwd 5′-GTT TGA T<C/T> <C/A> TGG CTC AG-3′ and 16SRev 5′-CA<T/G> AAA GGA GGT GAT CC-3′ (Horn et al., 2002). PCR conditions were 15 min at 94 °C, followed by 30 cycles of 94 °C for 30 s, 55 °C 30 s, 72 °C 2 min, with final extension of 72 °C for 5 min, except for the 16S PCR where the annealing temperature was increased to 58 °C. The resulting PCR products were sequenced by Edinburgh Genomics with the same primers as were used to amplify them plus internal primers where necessary.

2.4. Phylogenetic analysis

18S rDNA Sequences were obtained for genus *Leptomyxa* and relatives from Genbank and compiled together with the sequences

Table 2
Currently recognised members of the genus *Leptomyxa* (Smirnov et al., 2017; this study).

Leptomyxa species	Length (μm)	Uni or Multi nucleus	Cyst features & diameter (μm)	Food	Known environment	Reference
L. neglecta	70–140	Multinucleate 1 - 5	None described	Unknown	Freshwater lake	Smirnov et al., 2009
L. australiensis	50–180	Predominantly single	Single wall, no pores 15 - 40	Fungi & bacteria	Soil	Chakraborty and Pussard, 1985; Smirnov et al., 2017
L. reticulata	500–3000	Multinucleate	Double wall 35 - 60	Non-pigmented bacteria	Freshwater river	Goodey, 1914; Cann, 1984; Page 1988; Singh, 1948
L. arborea	50 ->1000	Multinucleate	Double wall multinucleate	Fungi yeast bacteria	Soil	Smirnov et al., 2017
L. variabilis	30–150	Multinucleate 1–20	Single wall with pores 15 -25	Unknown	Soil	Smirnov et al., 2017
L. valladaresi	70–180	Predominantly single	Single wall, no pores 19–28	amoebae *E. coli*	Soil	This study

obtained in this study using "Seaview", version 4 (Gouy et al., 2010). The program "BioEdit" (Hall, 1999) was used to edit sequences where needed and to determine levels of homology between sequences. Maximum likelihood phylogenetic trees were obtained using PhyML software (Guindon and Gascuel, 2003) using the GTR model implemented with Seaview version 4. The non-parametric analysis was performed with 100 bootstrap pseudo-replicates, using sequences from other Leptomyxids as the outgroup. Sequences obtained in this study were submitted to GenBank and are available under accession number KX792145 for the 18S rDNA gene, and MF441175 for the 16S rDNA endosymbiont sequence.

3. Results

Strain LLT was isolated by the usual "walk out" method in which amoeboid organisms crawl out from the sample on a lawn of (usually) *E. coli* (Neff, 1958). This encourages the isolation of small fast growing amoebae (most often *Acanthamoeba* and *Naegleria*). We noticed that a small number of large amoebae were feeding on the *Acanthamoeba* that preceded them and were able to isolate this amoeba by cloning. LLT was found to be reluctant to grow on agar surfaces presumably due to the very thin layer of water on the surface of these plates, however it was successfully isolated from such plates when *Acanthamoeba* sp was present. Initially it could not grow on *E. coli* when placed on *E. coli* spread non-nutrient agar plates, nor did it grow when presented with *E. coli* on flat bottomed flask in Neff's saline. However, after a year of growth on accompanying bacteria, LLT was found to be able to grow well on *E. coli* alone, with a mean generation time of 18 h. Although LLT was seen to phagocytose yeast when they were presented, neither *Pichia pastoris* nor *Rhodotorula mucilaginosa* alone could support the survival of this amoeba. It did however grow rapidly when placed with *Acanthamoeba* (Neff strain) in Neff's saline. A small vahlkampfiid amoebae, *Vermamoeba vermiformis* and *Vannella* were seen to be phagocytosed by LLT but as they were also in the presence of *E. coli* it was not clear if these amoebae alone could support the growth of LLT. LLT was also observed to phagocytose the hyphae of an unidentified fungus, however it was not clear if fungi alone could support the growth of LLT as this fungus overgrew cultures causing LLT to encyst.

Phylogenetic analysis of the 18S rDNA gene revealed that LLT groups with genus *Leptomyxa* (Fig. 4) and that it is most closely related to *L. variabilis*. The 18S rDNA sequence and the unique morphological features make it clear that LLT is a new species of *Leptomyxa* and we have named it *L. valladaresi*.

Monoaxenic cultures were established with axenically grown *Acanthamoeba*, by overnight treatment of LLT trophozoites and cysts with gentamycin (10 μg/ml) and ampicillin (100 μg/ml) in Neff's saline. These cultures were maintained at room temperature and supplemented with fresh *Acanthamoeba* when required. The

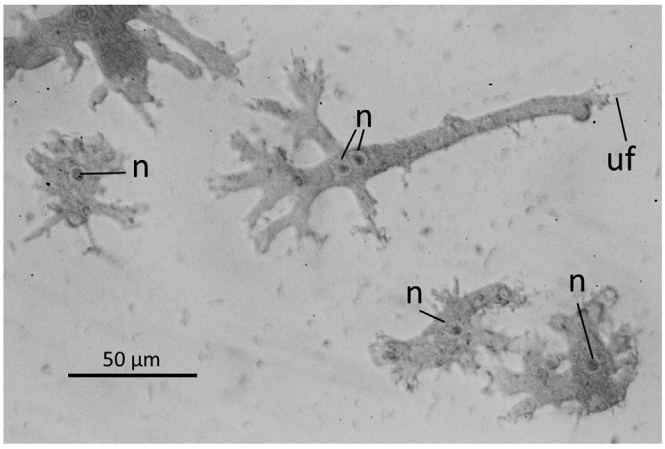


Fig. 1. Amoebae were fixed with Nissenbaum's fixative and the nuclei (n) were stained with Kernechtrot nuclear fast red. The uroidal filaments (uf) are also seen here.

LLT cysts are variable in size (Table 1, Fig. 2d) with a mean diameter of 23 μm. Most cysts contained a single nucleus, a few had double nuclei and fewer still contained three (Fig. 2d; Table 1). Perhaps as expected those with supernumerary nuclei tended to be larger, as is the case for other amoebae (De Jonckheere, 2006), yet not it seems for *Leptomyxa reticulata* (Cann, 1984). The trophozoites were very large as is typical for the genus (Table 2) and when locomotion was organized and rapid, short uroidal filaments were often visible (Fig. 1) and a tube shaped monopodal morphology adopted (Fig. 2b).

Overall the locomotory habit was similar to that described for other members of this order. The amoeba was variable in locomotory habit, changing rapidly for example from a fast moving cylindrical (limax) form with a single pseudopodium (Fig. 2b) to a flatter broader shaped form with a leading front which expanded on a number of different fronts often with changing dominance (Figs. 1 and 2a). The pseudopod into which the nucleus entered became dominant (Videos 2 and 3, see supplemental data) in a manner similar to that reported for *Balamuthia mandrillaris* (Dunnebacke, 2009). Nuclei drawn into these pseudopods were seen to deform as they did so, taking on temporary oval or cylindrical shapes. Simple monopodal forms were observed to stop and to travel in the opposite direction by the creation of a pseudopod from the former uroid in a manner similar as that described for other amoebae such as *Vahlkampfia inornata* (Page, 1967).

Supplementary video related to this article can be found at https://doi.org/10.1016/j.exppara.2017.09.017.

Forward advance often occurs by protrusion of broad, hyaline often hemispherical bulges which erupt forward in a manner which is normally considered to be a hallmark of the heterolobosean

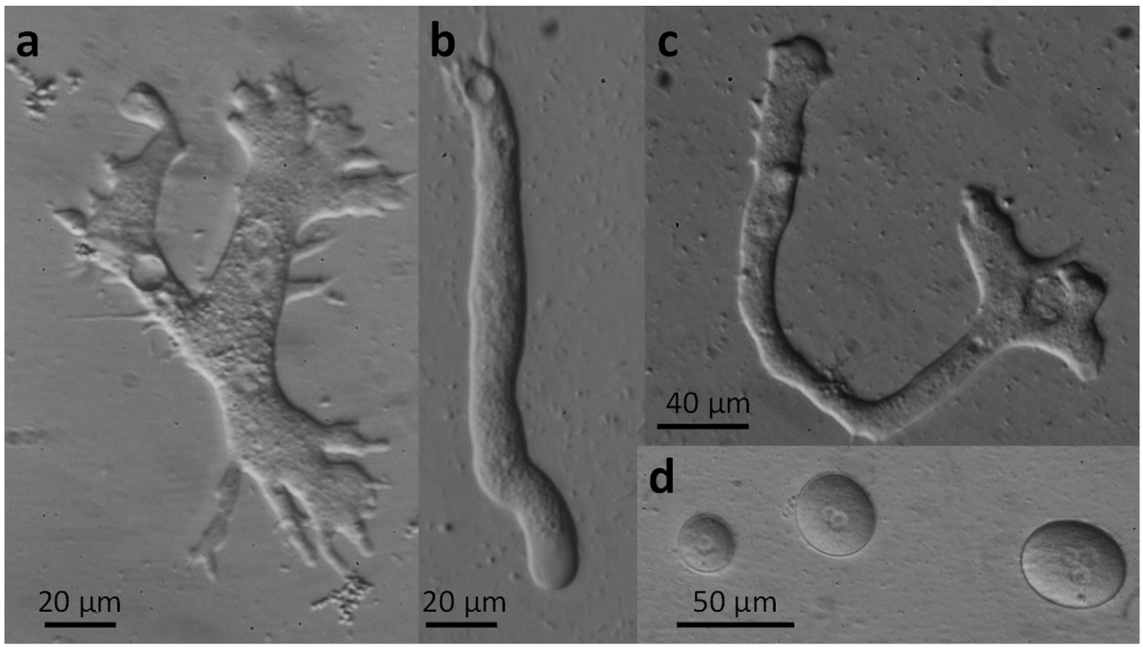


Fig. 2. a) Trophozoites in multi-pseudopod locomotion b) Trophozoite in limax locomotory form with the contractile vacuole (cv) toward the rear. d) Bipolar form with two leading pseudopods. c) Three cysts with one nucleus (left most cyst), two nuclei (middle) and three nuclei (right most cyst).

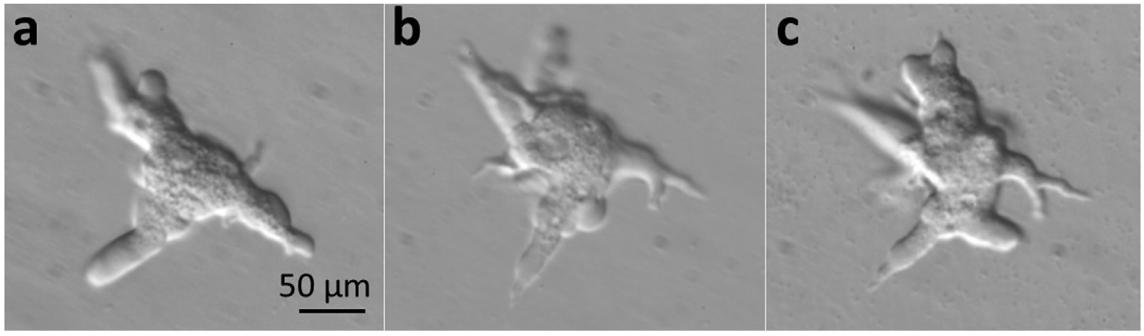


Fig. 3. Floating forms. 10 s between the three images showing the dynamic nature of the blunt pseudopods.

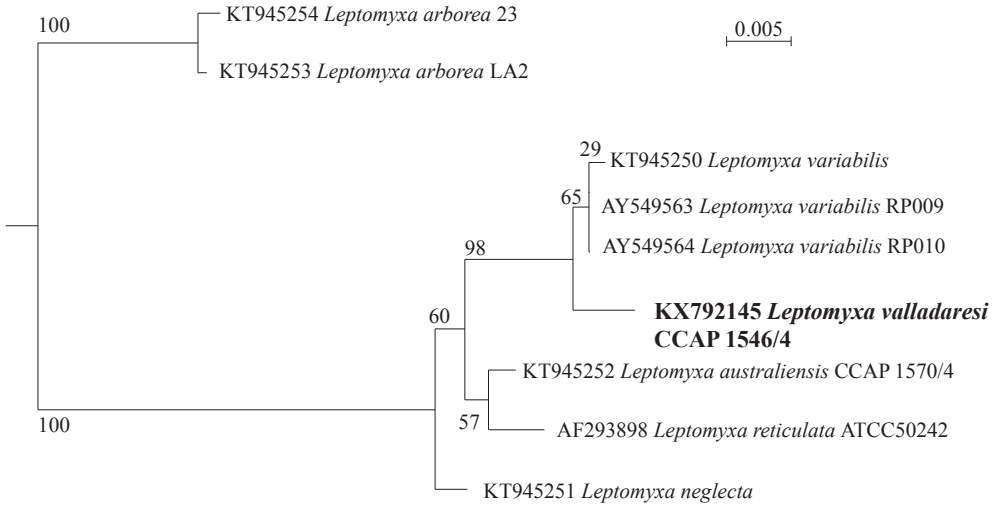


Fig. 4. Phylogenetic analysis 18S rDNA. PhyML ln(L) = -3625.7 1855 sites GTR 100 replication. 4 rate classes. Tree rooted using a number of related *Flabelulla* and *Rhizamoeba* as the outgroup.

amoebae. This similarity between some of the family Leptomyxidae and the class heterolobosea has of course been noted by others (Page, 1988) and it is suggested that some current amoebae presently labelled as *Rhizamoeba*, (such as *R. schnepfii*) may actually be heteroloboseans (Smirnov et al., 2009). Another shared feature is that the liquid hyaline ectoplasm tends to run down the side of the gelled endoplasmic cortex under the plasma membrane and then "setting", or becoming gelated. This has the appearance of candle wax spilling down the candle shaft and suddenly solidifying as it cools.

There was no particular floating form as described for *R. matisi* (Smirnov et al., 2017), instead amoebae kept in suspension for 2 h adopted a similar shape as they did will crawling but with blunt projections branching off the main body at all angles and in constant motion (Fig. 3). These quickly resumed locomotion as they settled on the vessel floor.

4. Discussion

In common with many groups of protozoans a congruence is sought between morphological features and specific sequence data in the genus *Leptomyxa* (Dyková et al., 2008; Smirnov et al., 2017). The genus *Leptomyxa* as it presently stands is composed of six named species and these are all terrestrial (soil/freshwater) members. The existence of further members is indicated by sequences from environmental samples (Smirnov et al., 2017). Despite the observed similarity in locomotory morphology between *Rhizamoeba*/*Leptomyxa* and the Heterolobosean, this genus is classified (Amaral Zettler et al., 2000; Tekle et al., 2008) as belonging to the family Leptomyxidae part of the Tubulinea clade within the super group 'Amoebozoa' together with well-known genera such as *Amoeba* and *Acanthamoeba*. The fact that the core *Rhizamoeba* strains group so closely with *Leptomyxa reticulata* is a puzzle since these organisms appear to be so very different at the light microscope level and in ultrastructure (Cann, 1984). As its name suggests *L. reticulata* is a reticulate amoeba often with hundreds of nuclei scattered throughout its many anastomosing pseudopodia (Cann, 1984; Page, 1988). Doubt has been expressed (Smirnov et al., 2009) if strain ATCC 50242 (Amaral Zettler et al., 2000) was correctly assigned to the species *Leptomyxa reticulata* since it is so dissimilar to other Leptomyxids. However, more recently, other species which have anastomosing reticulopodia have been isolated and 18S rDNA gene sequences analysis agrees that *L. reticulata* groups together with *L. arborea* (Smirnov et al., 2017).

An analysis of the available 18S sequences revealed that the *L. valladaresi* 18S gene is most similar to *L. variabilis* (Smirnov et al., 2017) which differs in only 2 nucleotides from the "*Ripidomyxa*" 18S gene. However, the situation is complex as the sequence deposited in Genbank (AY549563, AY549564) is not actually the isolate named as "*Ripidomyxa australiensis*" (Chakraborty and Pussard, 1985), although they seem to share very similar morphology (Hewett, 2006). Additionally, the *Rhizamoeba australiensis* available as CCAP 1570/4 is also a different isolate to either Australian isolate and in fact originated in roof gutter sediment in Melsbach, Germany. *Rhizamoeba australiensis* CCAP 1570/4 is now reclassified as *Leptomyxa australiensis* (Smirnov et al., 2017).

The generation time of 18 h compares with the 46–83-h generation of *Amoeba proteus* (Rogerson, 1980). 4 h for *Dictyostelium* (Fey et al., 2007) and for *Naegleria* (Chang, 1958), 6 h for *Acanthamoeba* (Jensen et al., 1970) *Mayorella* (90–160 μm in length) with generation times 41.6h (Laybourn-Parry et al., 1987). We found that there was a simple relationship between the length of an amoebal species and its generation time (Fig. 5). Such a relationship is to be expected as larger amoeba need to attain more mass than smaller amoebae in order to divide, and a similar relationship (generation

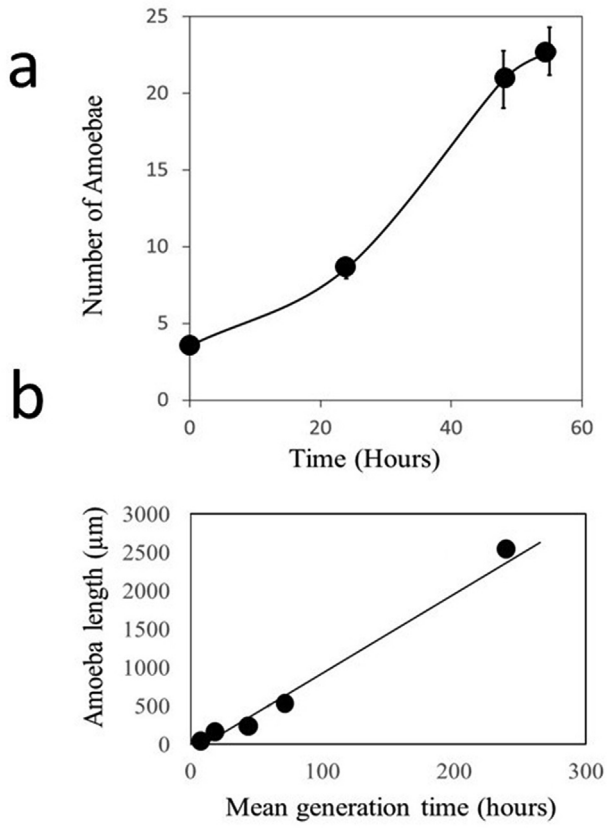


Fig. 5. a). The growth of *L. valladaresi* on *E. coli*. b). The mean generation time (GMT) of *L. valladaresi* compared to other amoebae (see main text) as a function of their length. A very simple relationship: GMT (hours) = Length (μm)/10, is apparent.

time = 0.311 Log $_{10}$ cell volume (μm^3) + 0.0445) has been reported (Baldock et al., 1980). The nuclei of *L. valladaresi* are large but usually single. Most other members of the genus have more than one nucleus presumably to produce the RNA required for cell growth and maintenance. The other function of the nucleus is to store the genome but there is no *a priori* reason the genome of *L. valladaresi* should be larger than a smaller amoeba such as *Acanthamoeba* which has a similarly unambitious life style. The (usually) single nucleus of *Amoeba dubia* has around 20,000 genome copies (Friz, 1968), and so is highly polyploid. Most amoebae reproduce asexually (Lahr et al., 2011), but asexual reproduction risks the accumulation of mutations by a process named "Muller's ratchet" (Muller, 1964). It has been argued that amoebae are polyploid and that together with recombination this prevents the operation of Muller's ratchet (Maciver, 2016). There is no evidence for sexual reproduction in *L. valladaresi* (or any species in this genus) and so it is likely that the large nucleus of this amoeba is polyploid to permit persistent asexual reproduction.

Leptomyxid amoebae have proven difficult to isolate and to maintain in culture (Smirnov et al., 2009). *L. valladaresi* thrives on *Acanthamoeba* as a food source and this di-axenic system is convenient to propagate this amoeba. However, it also grows very well on *E. coli*. The feeding habits of *L. valladaresi* are typical of amoebae of this size and it is not surprising that *Acanthamoeba*, *Vermamoeba* and other small amoebae serve as food. *Rhizamoeba* are reported to engulf protists (Rodriguez Zaragoza et al., 2005) and *Rhizamoeba matisi* is also known to consume *Vermamoeba vermiformis* (Smirnov et al., 2017). The original "*Ripidomyxa australiensis*" strain was also able to grow in the presence of a single bacterium (Chakraborty and Pussard, 1985), but this strain has been lost.

The observation that all cultures of *L. valladaresi* produce a geosmin-like smell independent of the food organisms present is similar to the case of the "*Ripidomyxa*" isolate (Robinson et al., 1995). The origin of geosmin was attributed to a symbiotic bacterium (Robinson et al., 1995; Hewett, 2006). 16S sequences from presumed endosymbiont bacteria from this strain (AY549554 and AY549555) had no matches in 2006 (Hewett, 2006), nor do they to date (July 2017). PCR using universal prokaryote16S primers revealed that *Delftia* or a related bacterium was present within *Leptomyxa valladaresi*. *Delftia* (a.k.a. *Comamonas*) is also known to infect *Acanthamoeba* (Walochnik et al., 1999). The production of geosmin by amoebae in culture has been reported in *Vannella* (Hayes et al., 1991). The significance of the production of geosmin is uncertain but volatile organic compounds affect amoeba prey selection (Schulz-Bohm et al., 2017), and it is possible that it has an anti-predator role (Höckelmann et al., 2009).

4.1. Diagnosis

Leptomyxa valladaresi n. sp. The amoeba adopts two main locomotory morphologies. It is transiently limax with a long, simple, tubular shape where the length is 70–180 μm; breadth 20–60 μm only occasionally does this form have an adhesive uroidal filaments or villous bulbous uroid. In limax locomotion the frontal hyaline cap occupies up to 1/5 of the total length of locomotive cell. The more usual locomotory habit is a flattened multi pseudopodal form and this form may have adhesive uroidal filaments and/or villous bulbous uroid but these are not as prominent a feature compared to reports for other leptomyxids. The amoeba is predominantly uninucleate but larger individuals may (rarely) have as many as 4 nuclei in old cultures. Nuclei are spherical 9 μm in diameter (range 6–11 μm). but can become oval shaped as they a compressed within a thinner part of the cell. The cyst (19–28 μm in diameter) is again usually uninucleate and is apparently single walled by light microscopy. The cysts can be stored cryogenically in the usual manner. There are no pores visible in the wall by light microscopy.

4.2. The type location

A dry gully in a large forested area consisting of the Canary Island Pine (*Pinus canariensis*) from a site known as "Las Lajas" (the rocks) on the southern flank of Mount Teide, Tenerife, Canary Islands, Spain. Site coordinates are lat.28.1886° long. −16.6644° altitude 2076 m above mean sea level. A fixed slide has been deposited with the Museum of Natural History, London (registration number NMHUK, 2016.9.16.1).

5. Differential diagnosis

Resembles L. *variabilis* in size and morphology, but *L. variabilis* is multicellular whereas *L. valladaresi* is predominantly mononucleate. It also resembles *L. australiensis* in size and organization of the locomotive form, and in the general size of the cyst (*L. valladaresi* being slightly smaller). In common with most Leptomyxid amoebae (Smirnov et al., 2017), a firm identification of *L. valladaresi* is only possible by sequencing the 18S gene.

6. Type material

The live strain is held at the Culture Collection of Algae and Protozoa at Oban, Argyle, Scotland, with the accession code CCAP 1546/4. 18S rDNA gene sequences obtained from the type strain were deposited with GenBank accession number KX792145.

7. Etymology

The species name *valladaresi* is to honour Professor Basilio Valladares on the occasion of his retirement after many years of dedicated service to the advancement of parasitology.

Acknowledgements

We are grateful to Dr Alexey Smirnov for making his work available to us prior to its publication. We also thank Dr Bret Robinson for very useful discussion of the "*Ripidomyxa*" isolate. The authors gratefully acknowledge support from Mexico's National Council of Science and Technology (CONACYT) and a University of Edinburgh, College of Medicine and Veterinary Medicine PhD Studentship. Support was also from Amoebics Ltd, Edinburgh.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.exppara.2017.09.017.

References

Amaral Zettler, L.A., Nerad, T.A., O'Kelly, C.J., Peglar, M.T., Gillevet, P.M., Silberman, J.D., Sogin, M.L., 2000. A Molecular reassessment of the Leptomyxid amoebae. Protist 151, 275–282.

Baldock, B.M., Baker, J.H., Sleigh, M.A., 1980. Laboratory growth rates of six species of freshwater Gymnamoebia. Oecologia 47, 156–159. https://doi.org/10.1007/BF00346813.

Bamforth, S.S., 2008. Protozoa of biological soil crusts of a cool desert in Utah. J. Arid. Environ. 72, 722–729.

Brown, S., Smirnov, A.V., 2004. Diversity of gymnamoebae in grassland soil in southern Scotland. Protistology 3 (3), 191–195.

Cann, J.P., 1984. The ultrastructure of *Rhizamoeba flabellata* (Goodey) comb. nov. and *Leptomyxa reticulata* Goodey (Acarpomyxea: Leptomyxida). Arch. Protistenk 128, 13–23.

Carter, R.F., 1970. Description of a *Naegleria* species isolated from two cases of primary amoebic meningoencephalitis and of the experimental pathological changes induced by it. J. Pathol. 100, 217–244.

Chakraborty, S., Pussard, M., 1985. *Ripidomyxa australiensis* nov. gen. nov. sp., a mycophagous amoeba from Australian soil. Protistologica 21, 133–140.

Chang, S.L., 1958. Cultural, cytological and ecological observations on the amoeba stage of *Naegleria gruberi*. J. Gen. Microbiol. 18, 565–578.

Corsaro, D., Venditti, D., 2010. Phylogenetic evidence for a new genotype of *Acanthamoeba* (Amoebozoa, Acanthamoebida). Parasitol. Res. 107, 233–238.

De Jonckheere, J., 2006. Isolation and molecular identification of Vahlkampfiid amoebae from an island (Tenerife, Spain). Acta protozool. 45, 91–96.

Dunnebacke, T.H., 2009. *Balamuthia mandrillaris*: the multiple nuclei of *Balamuthia* amebas; their location, activity, and site of development. Exp. Parasitol. 126 (1), 14–21.

Dyková, I., Fiala, I., Pecková, H., Dvořáková, H., 2008. Phylogeny of Flabellulidae (Amoebozoa: Leptomyxida) inferred from SSU rDNA sequences of the type strain of *Flabellula citata* Schaeffer, 1926 and newly isolated strains of marine amoebae. Folia Parasitol. 55, 256–264.

Fey, P., Kowal, A.S., Gaudet, P., Pilcher, K.E., Chisholm, R.L., 2007. Protocols for growth and development of *Dictyostelium discoideum*. Nat. Protoc. 2, 1307–1316.

Friz, C.T., 1968. The biochemical composition of the free living amoebae *Chaos chaos, Amoeba dubia* and *Amoeba proteus*. Comp. Biochem. Physiol. 26 (1), 81–90.

Gelman, B.B., Rauf, S.J., Nader, R., Popov, V., Borkowski, J., Chaljub, G., Nauta, H.W., Visvesvara, G.S., 2001. Amoebic encephalitis due to *Sappinia diploidea*. J. Amer.Med.Ass 285, 2450–2451.

Goodey, T., 1914. A preliminary communication of three new Proteomyxan Rhizopods from soil. Arch. Protistenk 35, 80–102.

Gouy, M., Guindon, S., Gascuel, O., 2010. SeaView version 4 : a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol. Biol. Evol. 27 (2), 221–224.

Guindon, S., Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52, 696–704.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser. 41, 95–98.

Hayes, S.J., Hayes, K.P., Robinson, B.S., 1991. Geosmin as an odorous metabolite in cultures of a free-living amoeba, *Vannella* species (Gymnamoebia, Vannellidae). J. Protozool. 38 (1), 44–47.

Hewett, M.K., 2006. Characterisation of Bacterial Symbionts in Amoebae. PhD Thesis. University of South Australia, Adelaide.

Höckelmann, C., Becher, P.G., von Reuß, S.H., Jüttner, F., 2009. Sesquiterpenes of the geosmin-producing Cyanobacterium *Calothrix* PCC 7507 and their toxicity to invertebrates. Z. Naturforsch 64, 49–55.

Horn, M., Fritsche, T.R., Linner, T., Gautom, R.K., Harzenetter, M.D., Wagner, M., 2002. Obligate bacterial endosymbionts of *Acanthamoeba* spp. Related to the *β-proteobacteria*: proposal of '*Candidatus probacter acanthamoebae*' gen. nov., sp. nov. Int. J. Syst. Evol. Microbiol. 52, 599–605.
Jensen, T., Barnes, W.G., Meyers, D., 1970. Axenic cultivation of large populations of *Acanthamoeba castellanii* (JBM). J. Parasitol. 56 (5), 904–906.
Kühn, S.F., 1996. *Rhizamoeba schnepfii* sp. nov., a naked amoeba feeding on marine diatoms (North Sea, German Bight). Arch. Protistenkd. 147, 277–282.
Lahr, D.J.G., Parfrey, L.W., Mitchell, E.A.D., Katz, L.A., Lara, E., 2011. The chastity of amoebae: re-evaluating evidence for sex in amoeboid organisms. Proc. Roy. Soc. B 278, 2081–2090.
Laybourn-Parry, J., Jones, K., Holdich, J.P., 1987. Grazing by *Mayorella* sp. (Protozoa: Sarcodina) on Cyanobacteria. Funct. Ecol. 1 (2), 99–104.
Lorenzo-Morales, J., Ortega-Rivas, A., Foronda, P., Martinez, E., Valladares, B., 2005. Isolation and identification of pathogenic *Acanthamoeba* strains in Tenerife, Canary islands, Spain from water sources. Parasitol. Res. 95, 273–277.
Lorenzo-Morales, J., Maciver, S.K., 2006. Long-term, low maintenance storage of *Acanthamoeba* strains. Parasitol. Res. 99 (6), 743–745.
Lorenzo-Morales, J., Khan, N.A., Walochnik, J., 2015. An update on *Acanthamoeba* keratitis: diagnosis, pathogenesis and treatment. Parasite 22, 10–20.
Maciver, S.K., 2007. The threat from *Balamuthia mandrillaris*. J. Med. Microbiol. 56, 1–3.
Maciver, S.K., Asif, M., Simmen, M.W., Lorenzo-Morales, J., 2013. A systematic analysis of *Acanthamoeba* genotype frequency correlated with source and pathogenicity: T4 is confirmed as a pathogen-rich genotype. Eur. J. Protistol. 49 (2), 217–221.
Maciver, S.K., 2016. Asexual amoebae escape Muller's ratchet through polyploidy. Trends Parasitol. 32 (11), 855–862.
Maciver, S.K., De Obeso Fernandez Del Valle, A., Koutsogiannis, Z., 2018. Vannella pentlandii n. sp., (Amoebozoa, Discosea, Vannellida) a small, cyst-forming soil amoeba. Exp. Parasitol. (submitted).
Mohamed, M.E., Huseein, E.A., Farrag, H.M., Mostafa, F.A.A., Hassan, A.T., 2016. *Allovahlkampfia spelaea* is a potential environmental host for pathogenic bacteria. J. Bacteriol. Parasitol. 7, 255.
Muller, H.J., 1964. The relation of recombination to mutational advance. Mutat. Res. 1, 2–9.
Neff, R.J., 1958. Mechanisms of purifying amoebae by migration on agar surfaces. J. Protozool. 5, 226–231.
Nissenbaum, G., 2001. Behind the discovery of "Nissenbaum's Fixative". J. Euk. Microbiol. 48 (1), 3–9.
Page, F.C., 1967. Taxonomic Criteria for limax amoebae, with descriptions of 3 new species of *Hartmannella* and 3 of *Vahlkampfia*. J. Protozool. 14 (3), 499–521.
Page, F.C., 1983. Marine Gymnamoebae. Institute of Terrestrial Ecology, Culture Centre of Algae and Protozoa (Cambridge, England).
Page, F.C., 1988. A New Key to Freshwater and Soil Gymnamoebae. CCAP. Freshwater Biological Ass. Scottish Marine Biol. Ass NERC.
Robinson, B., Hayes, K., Hayes, S., Dobson, P., 1995. 'Protozoa and their bacterial symbionts: a new group of organisms producing odorous metabolites'. In: Proceedings of the 7th International Conference on Small Free Living Amoebae (Adelaide).
Rodriguez Zaragoza, S., Mayzlish, E., Steinberger, Y., 2005. Seasonal changes in free-living amoeba species in the root canopy of *Zygophyllum dumosum* in the Negev Desert, Israel. Microb. Ecol. 49, 134–141.
Rogerson, A., 1980. Generation times and reproduction rates of *Amoeba proteus* (Leidy) as influenced by temperature and food concentration. Can. J. Zool. 58, 543–548.
Schuster, F.L., Visvesvara, G.S., 2004. Opportunistic amoebae: challenges in prophylaxis and treatment. Drug Resist Update 7, 41–51.
Schulz-Bohm, K., Geisen, S., Wubs, E.R.J., Song, C., de Boer, W., Garbeva, P., 2017. The prey's scent – volatile organic compound mediated interactions between soil bacteria and their protist predators. ISME J. 11, 817–820.
Shmakova, L.A., Fedorov-Davydov, D.G., Rivkina, E.M., 2013. The amoeboid protists of cryogenic soils in the Kolyma Lowland. Eurasian Soil S.C 46, 1211–1218.
Singh, B.N., 1948. Studies on giant amoeboid organisms 2. Nuclear division and cyst formation in *Leptomyxa reticdata* Goodey with remarks on the systematic position of the organism. J. Gen. Microbiol. 2 (1), 89–96.
Smirnov, A.V., Nassonova, E.S., Cavalier-Smith, T., 2008. Correct identification of species makes the amoebozoan rRNA tree congruent with morphology for the order Leptomyxida Page 1987; with description of *Acramoeba dendroida* n. g., n. sp., originally misidentified as 'Gephyramoeba sp'. Eur. J. Protistol. 44, 35–44.
Smirnov, A., Nassonova, E., Fahrni, J., Pawlowski, J., 2009. Rhizamoeba neglecta n. sp. (Amoebozoa, Tubulinea) from the bottom sediments of freshwater Lake Leshevoe (Valamo Island, North-Western Russia), with notes on the phylogeny of the order Leptomyxida. Eur. J. Protistol. 45, 251–259.
Smirnov, A., Nassonova, E., Geisen, S., Bonkowski, M., Kudryavtsev, A., Berney, C., Glotova, A., Bondarenko, N., Dykova, I., Mrva, M., Fahrni, J., Pawlowski, J., 2017. Phyogeny and systematics of Leptomyxid amoebae (Amoebozoa, Tubulinea, Leptomyxida). Protist 168, 220–252.
Tekle, Y.I., Grant, J., Roger Anderson, O., Nerad, T.A., Cole, J.C., Patterson, D.J., Katz, L.A., 2008. Phylogenetic placement of diverse amoebae inferred from multigene analyses and assessment of clade stability within 'Amoebozoa' upon removal of varying rate classes of SSU-rDNA. Mol. Phylo. Evol. 47 (1), 339–352.
Visvesvara, G.S., Martinez, A.J., Schuster, F.L., Leitch, G.J., Wallace, S.V., Sawyers, T.K., Anderson, M., 1990. Leptomyxid ameba, a new agent of amebic meningoencephalitis in humans and animals. J. Clin. Microbiol. 28, 2750–2756.
Visvesvara, G.S., Moura, H., Schuster, F.L., 2007. Pathogenic and opportunistic free-living amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris, Naegleria fowleri*, and *Sappinia diploidea*. FEMS Immunol. Med. Microbiol. 50, 1–26.
Walochnik, J., Picher, O., Aspöck, C., Ullmann, M., Sommer, R., Aspöck, H., 1999. Interactions of "Limax amoebae" gram-negative bacteria: experimental studies review of current problems. Tokai J. Exp.Clin. Med. 23 (6), 273–278.
Walochnik, J., Wylezich, C., Michel, R., 2010. The genus *Sappinia*: History, phylogeny and medical relevance. Exp. Parasitol. 126 (1), 4–13.

ACTA
PROTOZOOLOGICA

Acta Protozool. (2017) 56: 181–189
www.ejournals.eu/Acta-Protozoologica
doi:10.4467/16890027AP.17.016.7497

Allovahlkampfia minuta nov. sp., (Acrasidae, Heterolobosea, Excavata) a New Soil Amoeba at the Boundary of the Acrasid Cellular Slime Moulds

Alvaro DE OBESO FERNADEZ DEL VALLE, Sutherland K. MACIVER

Biomedical Sciences, Edinburgh Medical School, University of Edinburgh, Scotland, UK

Abstract. We report the isolation of a new species of *Allovahlkampfia*, a small cyst-forming heterolobosean soil amoeba. Phylogenetic analysis of the 18S rDNA and the internal transcribed spacers indicates that *Allovahlkampfia* is more closely related to the acrasids than to other heterolobosean groups and indicates that the new strain (GF1) groups with *Allovahlkampfia tibetiensis* and *A. nederlandiensis* despite being significantly smaller than these and any other described *Allovahlkampfia* species. GF1 forms aggregated cyst masses similar to the early stages of *Acrasis* sorocarp development, in agreement with the view that it shares ancestry with the acrasids. Time-lapse video microscopy reveals that trophozoites are attracted to individuals that have already begun to encyst or that have formed cysts. Although some members of the genus are known to be pathogenic the strain GF1 does not grow above 28°C nor at elevated osmotic conditions, indicating that it is unlikely to be a pathogen.

INTRODUCTION

The class heterolobosea was first created on morphological grounds to unite the schizopyrenid amoebae/amoeboflagellates with the acrasid slime moulds (Page and Blanton 1985), and subsequent molecular genetic data supported this union (Roger *et al*. 1996; Keeling and Doolittle 1996). The locomotory habit of these amoebae is to tend to produce an eruptive pseudopod, often alternatively from one side and then the other, of the advancing front. The heterolobosea are not directly related to other amoebae such as those within the Amoebozoa, despite being similar in appearance and habit. The heterolobosean acrasid slime moulds are very similar to the amoebozoan slime moulds too in life cycle, but these remarkable similarities in appearance and function are most probably due to parallel evolution.

It is not usually possible to classify the trophozoites of this group morphologically since they share very similar habits and appearances but many heterolobosean amoebae are also capable of transforming into flagellates, these are so called amoeboflagellates. In addition, some heterolobosean groups are comprised of flagellates only (*e.g*. *Percolomonas* and *Stephanopogon*) having apparently lost the ability to adopt the ancestral amoeboid habit. Heterolobosean amoebae which do not form flagellates were traditionally placed in the genus *Vahlkampfia* (Page 1976) but it has since been shown that this group is not monophyletic and other genera such as *Paravahlkampfia* and *Neovahlkampfia* were

Address for correspondence: Sutherland K. Maciver, Biomedical Sciences, Edinburgh Medical School, University of Edinburgh, Hugh Robson Building, George Square. Edinburgh. EH8 9XD, Scotland, UK; Phone: 44(0)131 650 3714; E-mail: smaciver@ed.ac.uk

formed (Brown and De Jonckheere 1999), to join other non-flagellate genera raised on morphological grounds. Further analysis of 18S RNA genes has allowed many other organisms to be included with the Heterolobosea and it is increasingly clear that it is an order with huge diversity (Pánek and Čepička 2016; Pánek *et al*. 2017). In 2009 it was necessary to raise a further genus, named *Allovahlkampfia* to place a new heterolobosean that did not fit elsewhere (Walochnik and Mulec 2009). A number of other isolates have now been added and as the genus *Allovahlkampfia* is presently configured, does not contain flagellates or amoeboflagellates but all members so far characterised, produce cysts without opercula. Members of *Allovahlkampfia* vary significantly in SSU rRNA sequence, with inserts present in some (Geisen *et al*. 2015). They are variable in size but the strain described in the present study which we name *Allovahlkampfia minuta*, is the smallest.

One heterolobosean amoeboflagellate, *Naegleria fowleri* is dangerously pathogenic for humans (Carter 1970; Siddiqui *et al*. 2016). Additionally, *Allovahlkampfia spelaea* is reported to infect the human eye (Huseein *et al*. 2016; Tolba *et al*. 2016) and to host bacteria known to be human pathogens (Mohamed *et al*. 2016) making this genus particularly worthy of study.

METHODS AND MATERIALS

Soil samples were collected from the Glenfinnan viaduct at the north end of Loch Shiel, Scotland, UK. Small quantities were settled on non-nutrient agar overlain with Neff's saline overnight. Strips of this were then inverted onto non-nutrient agar plates spread with *E. coli* (BL21) and monitored over a week for amoebal out growth. Small blocks were cut from the leading outgrowth onto fresh agar plates spread with *E. coli* until clones of strain GF1 were established. Large quantities of cloned GF1 amoebae were cultured on 2% agar YME plates containing 0.01% yeast extract and 0.025% malt extract overlain with Neff's saline at room temperature with *E. coli* as food organisms. For long term storage, the amoebae and cysts used in this study were taken up in Neff's saline with 10% DMSO and placed overnight in a freezer at –20°C, then transferred to –80°C freezer. Amoebae were also stored long term in clay pellets as previously described (Lorenzo-Morales and Maciver 2006).

To test tolerance to osmotic stress, amoebae were seeded on *E. coli* spread non-nutrient agar plates supplemented with 0.5 M, 1 M and 1.5 M mannitol as previously described (Khan *et al*. 2001). Temperature tolerance was also tested by seeding amoebae on *E. coli* spread non-nutrient agar plates incubated at various temperature and monitored for growth over a ten day period.

We investigated the ability of *A. minuta* to develop a flagellate stage with a variety of stimuli included the addition of distilled water, rapid changes in pH, CO_2 and O_2 levels in addition to physical agitation (Perkins and Jahn 1970).

DNA was isolated from GF1 as previously described (Lorenzo-Morales *et al*. 2005). Amoebae were lysed in buffer and treated with proteinase K at 60°C for 2 hours followed by phenol-chloroform extraction. DNA was then concentrated by precipitation with isopropanol. The resulting DNA was quantified using a "nanodrop" spectrophotometer (ThermoFisher). An aliquot of 100 ng of genomic DNA was used per PCR reaction, using GoTaq Green Master Mix polymerase (Promega).

Sequences were obtained from GenBank and other sources and compiled together with the new sequences from this study using "Seaview", version 4 (Gouy *et al*. 2010). "BioEdit" (Hall 1999) was used to trim sequences and to determine levels of homology between sequences. Sequences were aligned using seaview 4 which was also used to implement the PhyML algorithm to produce Maximum likelihood phylogenetic trees (Guindon and Gascuel 2003) using the GTR model. The non-parametric analysis was performed with 100 bootstrap pseudo-replicates, using representative sequences from other heterolobozoans (*Naegleria*, *Tetramitus* and *Vahlkampfia*) as the outgroup. Binomial names of the strains described previously (Geisen *et al*. 2015) were obtained from the CCAP website (https://www.ccap.ac.uk).

RESULTS

The locomotive form is noticeably smaller than that of previously described members of this genus (Table 1). It is often wider than it is long although this is usually temporary. Pseudopods are produced eruptively from the anterior in a manner typical of the heterolobosean amoebae (Fig. 1). The trophozoites can also adopt a flabellate-like morphology. In culture GF1 trophozoites and cysts were observed to collect at the water air boundary and migrated on the meniscus. Strain GF1 was found to produce aggregates of cysts on agar plates (Fig. 2). Time-lapse video microscopy (see supplementary data and Fig. 3) reveals that the aggregated nature of the cyst distribution results from trophozoites actively migrating toward preformed cysts and not as a result of cyst aggregating by a passive mechanism after formation. GF1 was found to grow at 4°C and slowly at 28°C but at 30°C it encysts. The optimal temperature for growth was 24°C. GF1 could not grow on plate supplemented with 0.5 M mannitol or above.

Phylogenetic analysis tentatively suggests that genus *Allovahlkampfia* is comprised of eight or nine groups, probably representing as many natural species. More data from more genes and isolates are required to clarify this of course. No flagellated cells have been found in culture despite the application of a range of previously reported flagellate inducing stimuli. Strain GF1 is closely related to two previously reported species (*A. nederlandiensis* and *A. tibetiensis*) (Fig. 4A)

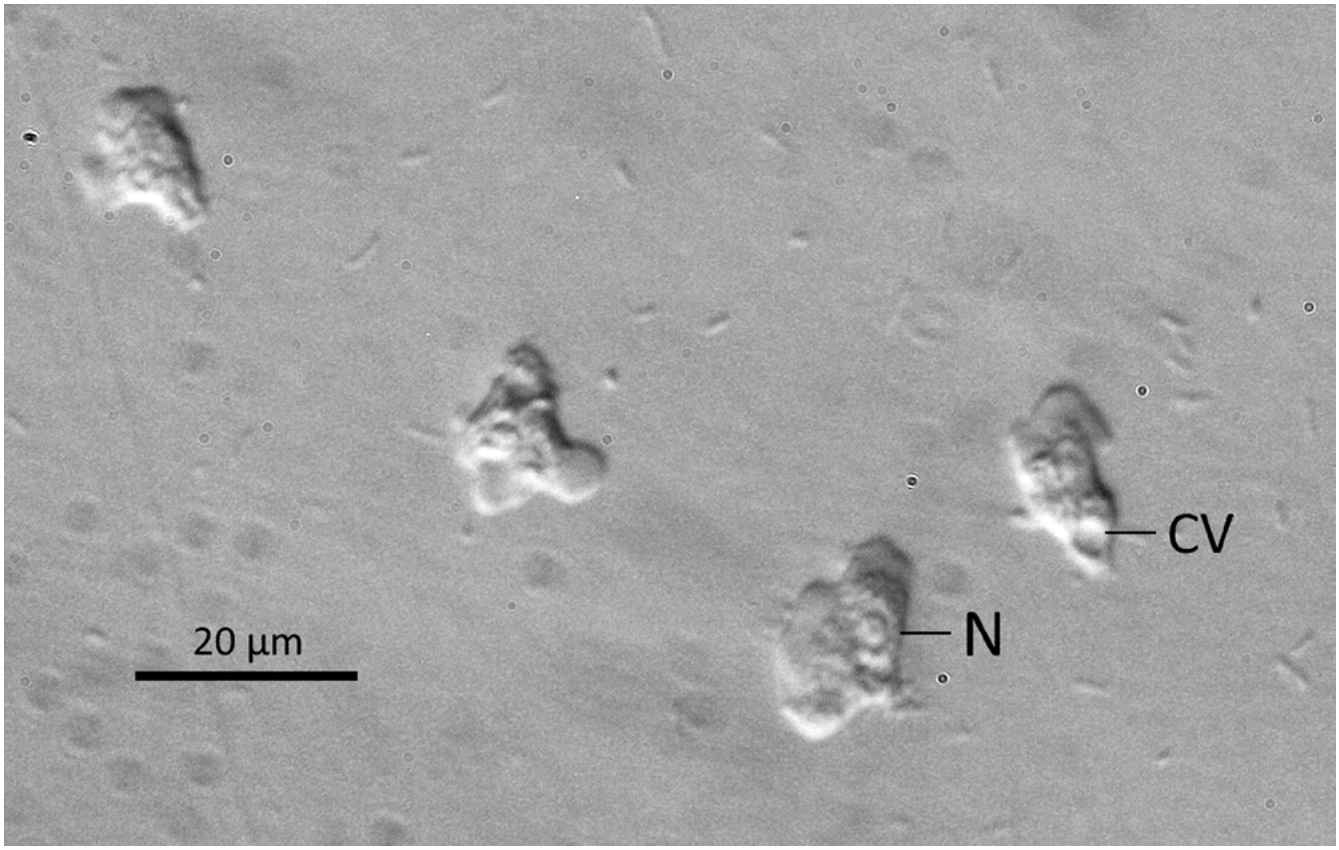


Fig. 1. Trophozoites of *Allovahlkampfia minuta* showing typical morphology with eruptive pseudopods. Nucleus (N) has a central nucleolus and the contractile vacuole (CV) is typically positioned at the rear of the amoeba. The scale bar is 20 µm. Food organisms, *E. coli* are visible in the background.

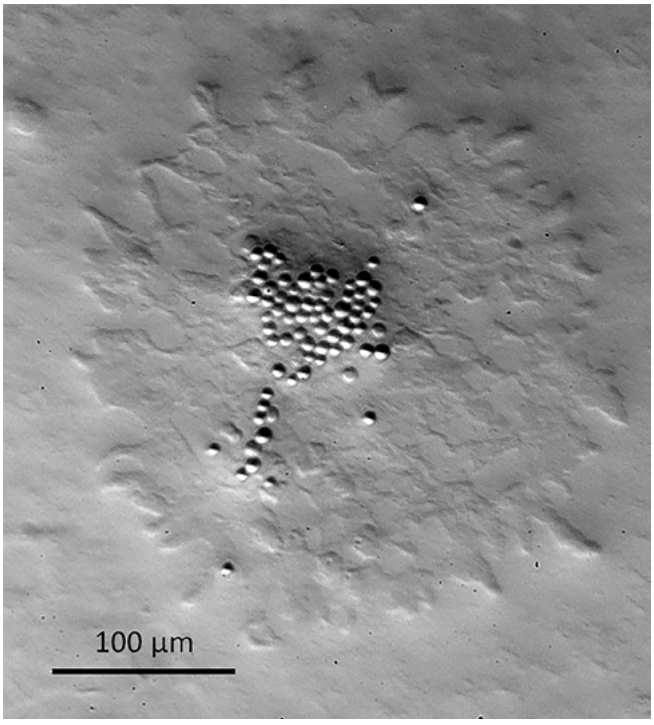


Fig. 2. Trophozoites gather and encyst on non-nutrient agar plate surface as the *E. coli* food organisms become depleted and as the conditions become less moist.

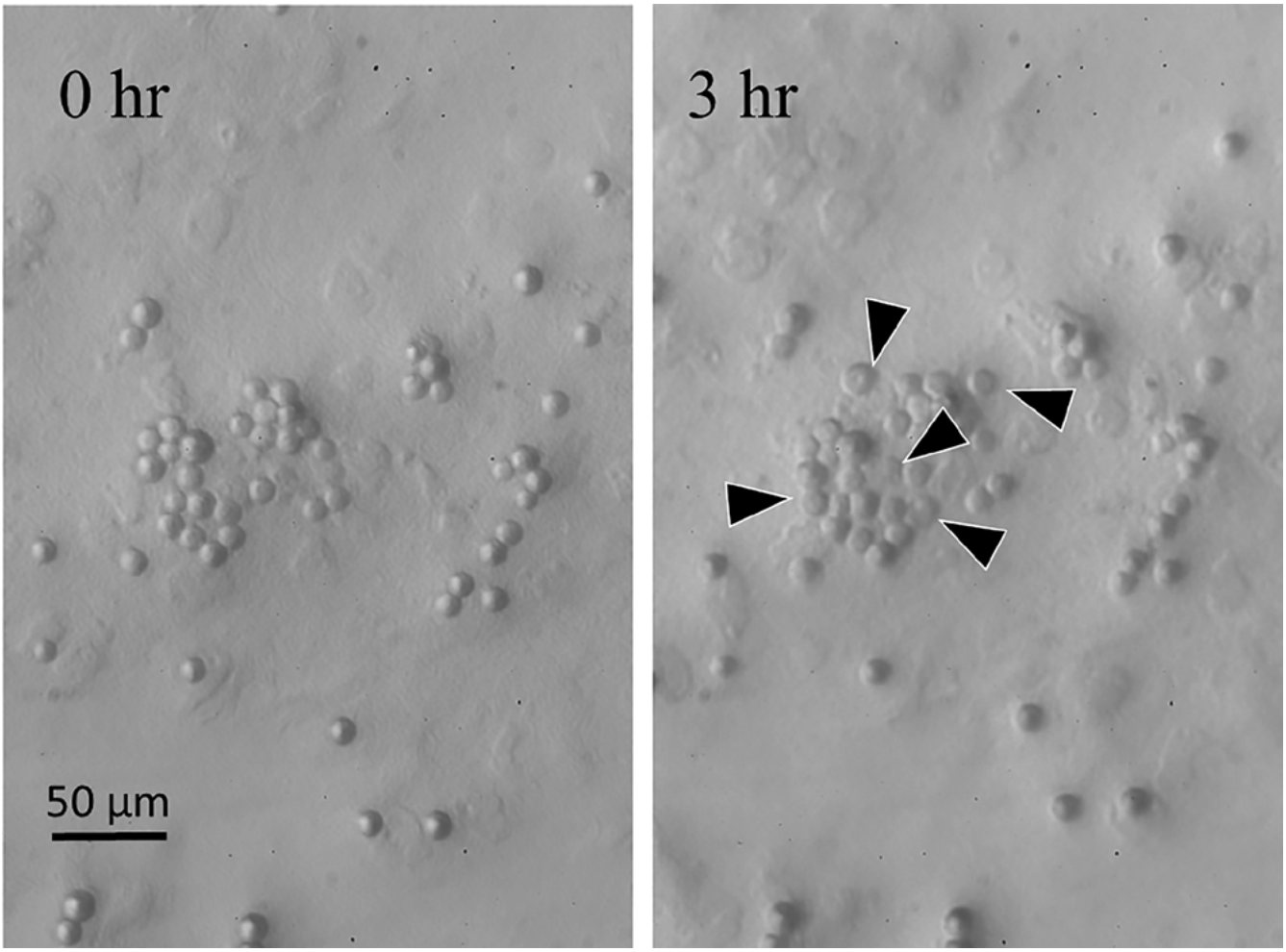


Fig. 3. Cooperative cyst formation. Trophozoites were placed on an agar plate with a monolayer of *E. coli*. Encystment took place when the plate became dry and the food source locally exhausted. Trophozoites aggregated around the first to encyst, forming a slightly raised mound of cysts. Left panel – This image is the first frame of a video (see supplementary data) taken to follow cyst aggregate formation. Right panel – This is the last image of the same video where black arrowheads point to cysts that have been produced during the time that the video was shot.

and these are very similar in appearance also, especially regarding the tendency to adopt a temporary flabellate locomotory habit. However, these other members of this group are significantly larger (Geisen *et al.* 2015) (Table 1).

DISCUSSION

The most notable feature of *A. minuta* is its small size but similarly sized heterolobosean amoebae have been described. For example, *Neovahlkampfia nana* is 12 µm in length (Tyml *et al.* 2017). Compared to other heteroloboseans including the genus *Allovahkmapfia*, the locomotion of *A. minuta* seems chaotic and inefficient with frequent direction changes and the production of seemingly immediately redundant pseudopods. *A. minuta* was observed to adopt a temporal flabellate morphology as is the case for other heteroloboseans for example *Heteramoeba clara* (Droop 1962) and *Vahlkampfia signyensis* (Garstecki *et al.* 2005). The habit of the cysts and trophozoites of *A. minuta* to collect at the meniscus has been reported to be the case with other free-living amoebae (Preston 2003).

The genus *Allovahlkampfia* is genetically diverse but morphologically uniform and represented so far only by freshwater and soil species. It is not possible to distinguish *Allovahlkampfia* from several other het-

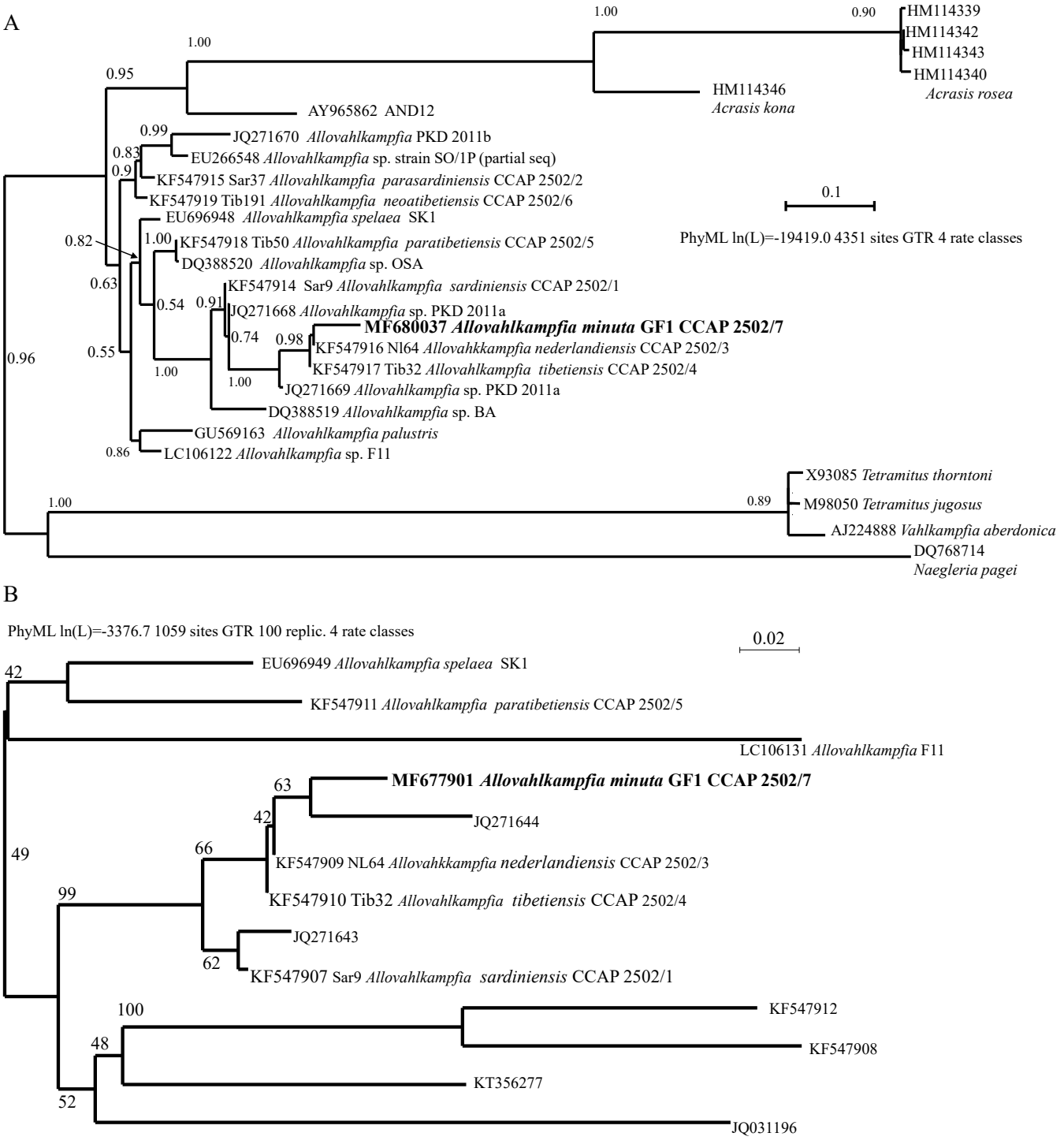


Fig. 4. A. A PhyML phylogenetic tree (GTR model) of members of some heterolobosean of the genus *Allovahlkampfia* and *Acrasis* based on 18S rDNA gene. Branch support values at each node indicated as percentages. The GenBank accession code of each sequence is followed by the species names, then strain name (where available). The tree has been rooted using a number of other heterolobosean genera (*Tetramitus*, *Naegleria* and *Vahlkampfia*) as the outgroup. Scale bar represents evolutionary distance. Binomials names for some strains (CCAP 2502/1 to 2502/6) were from the culture collection website (https://www.ccap.ac.uk) as they were not given in the original paper (Geisen *et al*. 2015). The subject of the present study is highlighted in bold. **B**. A PhyML phylogenetic tree (GTR model) of members of some heterolobosean of the genus *Allovahlkampfia* and *Acrasis* based on the fragment coding the internal transcribed spacer 1, 5.8S ribosomal RNA, and internal transcribed spacer 2.

Table 1

Species/strain	Length µm Average &/or range	Breadth µm Average & range	Cyst diameter µm Average &/or range	Reference
A. palustris	31 (14–43)	6.3 (4–12)	8–10	Anderson *et al*. 2011
A. spelaea	20–40	n/a	16–25	Walochnik and Mulec, 2009
AND12	20–30	n/a	n/a	Lara *et al.* 2007
A. tibetiensis	25.2 (21.2–31.2)	6.6 (5.6–9.2)	6.6 (5.0–8.2)	Geisen *et al.* 2015
A. nederlandiensis	33.2 (21.8–50.8)	5.8 (3.6–8.6)	6.2 (5.0–7.8)	Geisen *et al.* 2015
F11	35.8	11.4	11.5	Tyml *et al.* 2016
A. minuta	13.4 (9.5–16.7)	8.1 (4.6–15.6)	6.5 (4.7–8.9)	This study

erolobosean genera based on appearance alone (Geisen *et al*. 2015), even at the level of electron microscopy (González-Robles *et al*. 2012). The cysts of all presently named species of the genus *Allovahlkampfia*, including *A. minuta* described here, lack opercula (Anderson *et al*. 2011; Walochnik and Mulec 2009; Geisen *et al*. 2015). This is a feature shared by *Acrasis rosea* (Hohl and Hamamoto 1969). It is the small size of *A. minuta* that distinguishes it from others in the genus. None of the previously characterised members of the genus is close in size to the trophozoites or the cysts.

Since reports of morphological similarities between vahlkampfids and acrasids were published (Page 1978; Page and Blanton 1985), several authors have reported a close genetic affinity also (Keeling and Doolittle 1996; Roger et al. 1996). Comparing ribosomal genes allowed *Allovahlkampfia* and *Acrasis* to be placed in clade "Acrasidia" within the Tetramitia (De Jonckheere *et al*. 2011; Harding *et al*. 2013; Geisen *et al*. 2015; Pánek *et al*. 2017). In this connection, the Allovahlkampfid strain AND12 (Lara *et al*. 2007) is especially interesting as it is currently the most closely known relative of the genus *Acrasis*. However, it was only partially characterized and because it was observed in liquid culture, its ability to develop fruiting bodies is untested (Lara *et al*. 2007). Unfortunately, this strain is no longer available in culture (Ekelund, Pers. Comm), but amoebae at the boundary between *Allovahlkampfia* and *Acrasis* may reveal details on the development of sporogenesis.

The aggregation of *A. minuta* trophozoites during encystment, produces images very similar to those seen in the acrasids especially the morphologically simple *Acrasis helenhemmesae* (see figures 14–16 in Brown *et al*. 2010). This tendency to aggregate may be shared with others of the genus as *A. palustris* and *A. spelaea* cysts are also reported to occur in clumps, although no further information about this was given (Anderson *et al*. 2011; Tolba *et al.* 2016). The significance of this aggregation is not yet clear but we have found time lapse video evidence (see supplementary data) for chemotaxis towards the encysting centre. There may be increased survival of aggregated cysts, or the aggregation may, like is this case in the acrasids be a prelude to the production of Allovahlkampfid fruiting bodies that may form under as-yet unknown circumstances. In this regard, it is interesting to note that a simple linear fruiting body has been observed in *Allovahlkampfia* strain BA (Brown *et al*. 2012), however this was observed on only one occasion. Other hints that *Allovahlkampfia* can produce multicellular aggregates such as fruiting exist. The isolate, *Allovahlkampfia* strain OSA, has been described as "an orange stalk-like fungal growth habit" (Shutt 2006) and while this description is a little vague, it is compatible with it having a multi-cellular or at least aggregative stage. Confusingly however, neither BA nor OSA formed fruiting bodies according to the worker who isolated them (Schutt 2006). Strains BA and OSA were assigned to *A. spelaea* (Brown *et al*. 2012) but this suggestion has not been adopted by later workers (Geisen *et al.* 2015) and is not supported by our 18S rDNA analysis. The aggregation that we observed with encysting *A. minuta* amoeba is superficially similar to the aggregation during the encystment of the amoebozoan, *Vannella pentlandii* (Maciver *et al*. 2017). Also, the closely related *Vannella fimicola* produces an actual fruiting body (Olive, 1962). The ability to fruit has evolved apparently independently, in other amoebozoan groups, including *Luapeleamoeba hula* within family Acanthamoebidae (Shadwick *et al*. 2016), and surprisingly, including the genus *Acanthamoeba* itself (Tice *et al*. 2016). Amoebae other than the Amoebozoa

are also known to produce fruiting bodies (Kang *et al*. 2017). There is little evidence of sexual recombination in the acrasids (Brown *et al*. 2012), but it is likely that the purpose of these fruiting bodies in the acrasids may be a dispersive strategy carrying the spores off the substrate to be carried by air currents, or passing birds and insects.

Many genera of free living amoeba contain species pathogenic to humans and while most of these including *Acanthamoeba*, *Balamuthia* and *Sappinia* are members of the amoebozoa some are heteroloboseans. *Acanthamoeba* is notorious for causing *Acanthamoeba* keratitis (Lorenzo-Morales *et al.* 2015), a sight threatening and extremely painful infection of the eye but there are reported cases of a similar keratitis associated with the presence of amoebae tentatively identified as being *Vahlkampfia* but in the presence of other organisms (Aitken *et al.*1996; Alexandrakis *et al*. 1998; Niyyati *et al*. 2010; González-Robles *et al*. 2012). These reports, while not concluding a directly causal link, do reveal that that heteroloboseans can survive on the human eye. Stronger evidence suggests that an amoeba SO/1P now known to belong in genus *Allovahlkampfia* (Fig. 2), cause keratitis in humans with herpes virus (Ozkoc *et al*. 2008) and that this same strain produced a similar infection in rat models (Huseein *et al*. 2016) (presumably in the absence of herpes virus since it was not mentioned). A second case where an amoeba closely related to *Allovahlkampfia spelaea* was reported to cause keratitis has also been reported (Tolba *et al*. 2016) and this strain also caused keratitis in a rabbit model. Whereas 93% of *Acanthamoeba* keratitis cases are associated with contact lens use (Radford *et al*. 1998), the few cases reported for heterolobosean amoebae have been dominated by trauma to the eye surface (Alexandrakis *et al*. 1998; Ozkoc *et al*. 2008; Tolba *et al.* 2016). It is firmly established that another heterolobosean, *Naegleria fowleri* causes the usually fatal brain infection primary amoebic meningoencephalitis in humans (Siddiqui *et al*. 2016). The finding that *A. minuta* does not grow above 28°C, and its failure to cope with elevated osmolarity, would seem to rule it out as a potential human eye pathogen where the temperature varies between 32 and 36°C (Purslow *et al.* 2005).

Allovahlkampfia minuta n. sp.

Description. The locomotive morphology of this species is typical for small heterolobosean amoebae. Amoeba length 13.4 µm (range 9.5–16.7) and width 8.1 µm (range 4.6–15.6). Length:breadth ratio is 1.65. A well-developed uroid is not often visible but trailing uroidal filaments are occasionally observed on glass and tissue culture plastic. The spherical nucleus is variable in diameter but it ranges between 1.5–2.5 µm. There is a central nucleolus. A contractile vacuole is usually visible in trophozoites in Neff's saline or distilled water. This is usually located at the rear of the amoeba, often within the bulbous uroid, if it is present. Growth on *E. coli* is observed between 4 and 28°C, with an optimum at 24°C. The pelleted trophozoites have a distinct pink/orange colour.

Cysts. Spheroidal diameter 6.5 µm (range 4.7–8.9) with a single cell wall and lacking pores. The mononuclear cysts are usually formed in aggregates.

Etymology. This isolate is significantly smaller than any other described *Allovahlkampfia* species, hence the name ''*minuta*''.

Type locality. Bank of Loch Shiel (freshwater) near the Glennfinnan monument (latitude 56.8687; longitude – 5.4378), Scotland, UK. The elevation is 4 m.

Habitat. Alluvial soil.

Type material. A culture of *A. minuta* has been deposited with the Culture Collection of Algae and Protists, accession number CCAP 2502/7, and gene sequences deposited with GenBank, MF680037 and MF677901 for the 18S rRNA gene and the ITS region respectively.

Acknowedgements. We are grateful to Mr Samuel Casasola Zamora for collecting soil samples. We are pleased to acknowledge support from Mexico's National Council of Science and Technology (CONACYT) and a University of Edinburgh College of Medicine and Veterinary Medicine PhD Studentship. Support from Amoebics Ltd, Edinburgh is also acknowledged. We also gratefully acknowledge two anonymous reviewers for their useful and insightful comments on an earlier version of the manuscript.

REFERENCES

Aitken D., Hay J., Kinnear F. B., Kirkness C. M., Lee W. R., Seal D.V. (1996) Amebic keratitis in a wearer of disposable contact lenses due to a mixed *Vahlkampfia* and *Hartmanella* infection. *Ophthalmol.***103:** 485–494

Anderson O. R., Wang W. E. N., Faucher S. P., Bi K., Shuman H. A. (2011) A new heterolobosean amoeba *Solumitrus palustris* n. g., n. sp. isolated from freshwater marsh soil. *J. Eukaryot. Microbiol.* **58:** 60–67

Alexandrakis G., Miller D., Huang A. J. W. (1998) Amebic keratitis due to *Vahlkampfia* infection following corneal trauma. *Arch. Ophthalmol.* **117:** 950–951

Brown S., De Jonckheere J. F. (1999) A reevaluation of the amoeba genus *Vahlkampfia* based on SSUrDNA sequences. *Eur. J. Protistol*. **35:** 49–54

Brown M. W., Silberman J. D., Spiegel F. W. (2010) A morphologically simple species of *Acrasis* (Heterolobosea, Excavata), *Acrasis helenhemmesae* n. sp. *J. Eukaryot. Microbiol*. **57:** 346–353

Brown M. W., Silberman J. D., Spiegel F. W. (2012) A contemporary evaluation of the acrasids (Acrasidae, Heterolobosea, Excavata). *Eur. J. Protistol.* **48:** 103–123

Carter R. F. (1970) Description of a *Naegleria* species isolated from two cases of primary amoebic meningoencephalitis and of the experimental pathological changes induced by it. *J. Pathol.* **100:** 217–244

De Jonckheere J. F., Murase J., Opperdoes F. R. (2011) A new thermophilic heterolobosean amoeba, *Fumarolamoeba ceborucoi*, gen. nov., sp. nov., isolated near a fumarole at a volcano in Mexico. *Acta Protozool.* **50:** 41–48

Droop M. R. (1962) *Heteramoeba clara* n. gen., n. sp., a sexual biphasic amoeba. *Arch. Microbiol.* **42:** 254–266

Garstecki T., Brown S., De Jonckheere J. F. (2005) Description of *Vahlkampfia signyensis* n. sp. (Heterolobosea), based on morphological, ultrastructural and molecular characteristics. *Eur. J. Protistol.* **41:** 119–127

Geisen S., Bonkowski M., Zhang J., De Jonckheere J. F. (2015) Heterogeneity in the genus *Allovahlkampfia* and the description of the new genus *Parafumarolamoeba* (Vahlkampfiidae; Heterolobosea). *Eur. J. Protistol.* **51:** 335–349

González-Robles A., Salazar-Villatoro L., González-Lázaro M., Omaña-Molina M., Martínez-Palomo A. (2012) *Vahlkampfia* sp: Structural observations of cultured trophozoites. *Exp. Parasitol.* **130:** 86–90

Gouy M., Guindon S., Gascuel O. (2010) SeaView Version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol. Biol. Evol.* **27:** 221–224

Guindon S., Gascuel O., (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst. Biol.* **52:** 696–704

Hall T. A., (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids Symp. Ser.* **41:** 95–98

Harding T., Brown M. W., Plotnikov A., Selivanova E., Park J. S., Gunderson J. H., Baumgartner M., Silberman J. D., Roger A. J., Simpson A. G. (2013) Amoeba stages in the deepest branching heterolobosеans, including *Pharyngomonas*: evolutionary and systematic implications. *Protist* **164:** 272–286

Hohl H. R., Hamamoto S. T. (1969) Ultrastructure of *Acrasis rosea,* a cellular slime mold, during development. *J. Protozool.* **16:** 333–344

Huseein E. A. M., Anwer F. E. S., Abed G. H., Abdel-Gahfar K. S., Omar H. E. D. M. (2016) Keratitis induced by *Allovahlkampfia spelaea* in experimental rat model: a trial for treatment with ellagic acid. *Eur. J. Biol. Res.* **6:** 299–309

Kang S., Tice A. K., Spiegel F. W., Silberman J. D., Pánek T., Čepička I., Kostka M., Kosakyan A., Alcântara D. M., Roger A. J., Shadwick L. L., Smirnov A., Kudryavstev A., Lahr D. J. G., Brown M. W. (2017) Between a pod and a hard test: the deep evolution of amoebae. *Mol. Biol.Evol.* **34:** 2258–2270

Keeling P. J., Doolittle W. F. (1996) Alpha-tubulin from early diverging eukaryotic lineages and the evolution of the tubulin family. *Mol. Biol. Evol*. **13:** 1297–1305

Khan N. A., Jarroll E. J., Paget T. A. (2001) *Acanthamoeba* can be differentiated by the polymerase chain reaction and simple plating assays. *Curr. Microbiol.* **43:** 204–208

Lara E., Berney C., Ekelund F., Harms H., Chatzinotas A. (2007) Molecular comparison of cultivable protozoa from a pristine and a polycyclic aromatic hydrocarbon polluted site. *Soil Biol. Biochem.* **39:** 139–148

Lorenzo-Morales J., Lindo J. F., Martinez E., Calder D., Figueruelo E., Valladares B., Ortega-Rivas A. (2005) Pathogenic *Acanthamoeba* strains from water sources in Jamaica, West Indies. *Ann. Trop. Med. & Parasitol*. **99:** 751–758

Lorenzo-Morales J., Maciver S. K. (2006) Long-term, low maintenance storage of *Acanthamoeba* strains. *Parasitology Research* **99:** 743–745

Lorenzo-Morales J., Khan N. A., Walochnik J. (2015) An update on *Acanthamoeba* keratitis: diagnosis, pathogenesis and treatment. *Parasite* **22:** 10–20

Maciver S. K., De Obeso Fernandez Del Valle A., Koutsogiannis Z. (2017) *Vannella pentlandii* n. sp., (Amoebozoa, Discosea, Vannellida) a small, cyst-forming soil amoeba. *Exp. Parasitol*. *(in press)*

Mohamed M. E., Huseein E. A., Farrag H. M., Mostafa F. A. A., Hassan A. T. (2016) *Allovahlkampfia spelaea* is a potential environmental host for pathogenic bacteria. *J. Bacteriol. Parasitol.* **7:** 255–262

Niyyati M., Lorenzo-Morales J., Rezaie S., Rahimi F., Martín-Navarro C. M., Mohebali M., Rezaeian M. (2010) First report of a mixed infection due to *Acanthamoeba* genotype T3 and *Vahlkampfia* in a cosmetic soft contact lens wearer in Iran. *Exp. Parasitol.* **126:** 89–90

Olive L. S. (1962) The genus *Protostelium*. *Amer. J. Botany* **49:** 297–303

Ozkoc S., Tuncay S., Delibas S. B., Akisu C., Ozbek Z., Durak I., Walochnik J. (2008) Identification of *Acanthamoeba* genotype T4 and *Paravahlkampfia* sp. from two clinical samples. *J. Med. Microbiol.* **57:** 392–396

Page F. C. (1976) An illustrated key to freshwater and soil amoebae. Freshwater Biological Association, Ambleside, England.

Page F. C. (1978) *Acrasis rosea* and the possible relationship between Acrasida and Schizopyrenida. *Archiv für Protistenkunde.* **120:** 169–181

Page F. C., Blanton R. L. (1985) The Heterolobosea (Sarcodina:Rhizopoda), a new class uniting the Schizopyrenida and the Acrasidae (Acrasida). *Protistologica* **21:** 121–132

Pánek T., Čepička I. (2016) Diversity of Heterolobosea. In "Genetic Diversity of Microorganisms", 3–26. Eds. Caliskan M., Rijeka M : InTech.

Pánek T., Simpson A. G. B., Brown M. W., Dyer B. D. (2017) Heterolobosea in "Handboook of the Protists" Archibald *et al.*, eds Springer Int. Publ.

Perkins D. L., Jahn T. L. (1970) Amoeboflagellate transformations and the Gibbs-Donnan ratio. *J. Protozool.* **17:** 168–172

Preston T. M. (2003) The water-air interface: a microhabitat for amoebae. *Eur. J. Protistol*. **39:** 385–389

Purslow C., Wolffsohm J. S., Santodomingo-Rubido J. (2005) The effect of contact lens wear on dynamic ocular surface temperature. *Contact Lens & Ant. Eye* **28:** 29–36

Radford C. F., Lehmann O. J., Dart J. K. (1998) *Acanthamoeba* keratitis: multicentre survey in England 1992–1996. *Br. J. Ophthalmol.* **82:** 1387–1392

Roger A. J., Smith M. W., Doolittle R. F., Doolittle W. F. (1996) Evidence for the heterolobosea from phylogenetic analysis of genes encoding glyceraldehyde-3-phosphate dehydrogenase. *J. Euk. Microbiol.* **43:** 475–485

Shadwick L. L., Brown M. W., Tice A. K., Spiegel F. W. (2016) A new amoeba with Protosteloid Fruiting: *Luapeleamoeba hula* n. g. n. sp. (Acanthamoebidae, Centramoebida, Amoebozoa). *Acta Protozool.* **55:** 123–134

Shutt T. E. (2006) Origins and evolution of mitochondrial transcription and replication machinery. Ph.D. Thesis, Dalhousie University, Halifax, Nova Scotia.

Siddiqui R., Ali K. M., Cope J. R., Khan N. A. (2016) Biology and pathogenesis of *Naegleria fowleri*. *Acta Tropica* **164:** 375–394

Tice A. K., Shadwick L. L., Fiore-Donno A. M., Geisen S., Kang S.,Schuler G. A.,Spiegel F. W., Wilkinson K. A., Bonkowski M., Dumack K., Lahr D. J. G., Voelcker E., Clauß S., Zhang J. Brown M. W. (2016) Expansion of the molecular and morphological diversity of Acanthamoebidae (Centramoebida, Amoebozoa) and identification of a novel life cycle type within the group. *Biology Direct* **11:** 69; doi 10.1186/s13062-016-0171-0

Tolba M. E. M., Huseein E. A. M., Farrag H. M. M., Mohamed H. E. D., Kobayashi S., Suzuki J., Sugano S. (2016) *Allovahlkampfia spelaea* causing keratitis in humans. *PLoS Negl. Trop. Dis.* **10:** e0004841

Tyml T., Skulinová K., Kavan J., Ditrich O., Kostka M., Dyková I. (2016) Heterolobosean amoebae from Arctic and Antarctic extremes: 18 novel strains of *Allovahlkampfia*, *Vahlkampfia* and *Naegleria. Eur. J. Protistol.* **56:** 119–133

Tyml T., Lares-Jiménez L. F., Kostka M., Dyková I. (2017) *Neovahlkampfia nana* n. sp. Reinforcing an underrepresented subclade of Tetramitia, Heterolobosea. *J. Euk. Microbiol.* **64:** 78–87

Walochnik J., Mulec, J. (2009) Free-living amoebae in carbonate precipitating microhabitats of karst caves and a new vahlkampfiid amoeba, *Allovahlkampfia spelaea* gen. nov., sp. nov. *Acta Protozool*. **48:** 25–33

Received on 10th August, 2017; revised on 3rd October, 2017; accepted on 8th October, 2017

Supplementary data available on journal website

Video 1. Locomotion of *Allovahlkampfia minuta* trophozoites. Real time video micrography shows typical eruptive locomotion but also show the chaotic nature of the progression of this species.

Video 2. Cyst chemotaxis. Time lapse video with frames every 15 seconds, total time 3 hours. While some trophozoites continue to move feed and divide, others are drawn toward existing cysts and they too then encyst. Contractile vacuole activity gradually lessens as cyst development takes place.

Appendix 2. Primers

ASA.S1 (Dyková et al., 1999)
JDP1
GGCCCAGATCGTTTACCGTGAA
JDP2
TGACTCCCCTAGCAGCTTGTGAGA
Full 18S (Corsaro et al., 2015; Weekers et al., 1994)
Forward
GACTGGTTGATCCTGCCAG
Reverse
TGATCCTTTCGCAGGTTCAC
1137 Mid primer Reverse
ATTGACGGAAGGGCAC
Cytochrome Oxydase 1 COI (Nassonova et al., 2010)
Forward
GGTCAACAAATCATAAAGATATTG
Reverse
ACTAAAGATCTTTATACCAGTTGG
16S (Horn et al., 1999)
Forward
AGAGTTTGATYMTGGCTCAG
Reverse
CAKAAAGGAGGTGATCC
Protease 33 Gateway
Forward
GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGCGCGCCTACTACCTT
Reverse
GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAGCACCTGGCGGTGTA
Protease 33 aLICator
Forward
GGTTGGGAATTGCAAATGCGTGCCTACTTCGTGG
Reverse
GGAGATGGGAAGTCATTACTAGCACCGGGCGGAGTA
Lysozyme Gateway
Forward
GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAAGTCCACCTCCGTTCTTGT
Reverse
GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGTTGTCGGGGTACCAGT
Lysozyme aLICator
Forward
GGTTGGGAATTGCAAATGAAGTCCACCTCCGTTCTTGT
Reverse
GGAGATGGGAAGTCATTATCAGTTGTCGGGGTACCAGT

Appendix 3. Sequences obtained from isolated strains

Neff 18S (*Acanthamoeba*)

GGCTAACTAGACTAGCCATGCAGTGTAAGTATAAACTGCATTATACAGGTGAACCTGCG
AAAGGATCAACCCGTCAATGAGCATGTTCACAAGTGGGCTTATGAAGTTTTCATGCTCCT
TGCGGAACCTTGGTAATTCTTCAGCTAATAGTGCGCAAGGTCCCGAGCGCGGGGGGCAG
GGCTTCACGGCCCTGTCCTCGCATGCGCAGAGGGATGTATTTATTAGGTTAAAAACCAGC
GGCAGGGGTCAGCAATGGCCCCTGCCAAACACTCCTGGTGATTCATAGTAACTCTTTCGG
ATCGCATTCATGTCCTCCTTGTGGGGACGGCGACGATTCATTCAAATTTCTGCCCTATCAA
CTTTCGATGGTAGGATAGAGGCCTACCATGGTCGTAACGGGTAACGGAGAATTAGGGTT
CGATTCCGGAGAGGGAGCCTGAGAAATGGCTACCACTTCTAAGGAAGGCAGCAGGCGCG
CAAATTACCCAATCCCGACACGGGGAGGTAGTGACAATAAATAACAATACAGGCGCTCG
ATAAGAGTCTTGTAATTGGAATGAGTACAATTTAAACCCCTTAACGAGTAACAATTGGAG
GGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGT
TGCAGTTAAAAAGCTCGTAGTTGGATCTAGGGACGCGCATTTCAAGCGCCCGTGTCGTCG
GGTCAAACCGGCGACTGCGTTGGCGTTGCGGGCTCGGTCCGTCGGTGGACCCTCGTGGTC
TTAATCGGCGTGTCAACCGGCCCGCCCGTCCCCTCCTTCTGGATTCCCGTTCCTGCTATTG
AGTTAGTGGGGACGTCACAGGGGGCTCATCGTCGTCATGCAAATGGCGGCGGTGGGTCC
CTGGGGCCCAGATCGTTTACCGTGAAAAAATTAGAGTGTTCAAAGCAGGCAGATCCAAT
TTTCTGCCACCGAATACATTAGCATGGGATAATGGAATAGGACCCTGTCCTCCTATTTTC
AGTTGGTTTTGGCAGCGCGAGGACTAGGGTAATGATTAATAGGGATAGTTGGGGGCATT
AATATTTAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGATTAACTTCTGCGAAAGC
ATCTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAG
ATACCGTCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATAC
AAAACACCACCATCGGCGCGGTCGTCCTTGGCGTCTGTCCCTTTCAACGGGGGCAGGCGC
GAGGGCGGTTTAGCCCAGTGGCAGCGGTGAATGACTCCCCTAGCAGCTTGTGAGAAATC
ATAAGTCTTTGGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGGAATTGAC
GGAAGGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTT
ACCAGGTCCGGACATAGTAAGGATTGACAGATTGATAGCTCTTTCTTGATTCTATGGGTG
GTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGTTAACGAAC
GAGACCTTAACCTGCTAAATATGCCGCGCTAACCCGTCCATCAAAACCCATGCGTGGCTC
ACGCGGTCCGCTGCGGGGTGGTGTCGCTTCGCGGCGACGTCATCCCGCCGGCAGGGCCC
GGGTCCGTGTGGGCGGTAGGGTTCGGCGTCCGTGCTTCTTAGAGGGACTGCTGCGCGCCT
AGCCAGCGGAAGTTTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCA
CGCGCGCTACACTGATTAATCCAACGAGTCCGCTTCAATCGAGGCGCGATGCCGTTGGGG
TCAAACCCAACTGTGTCGCTGTCCTCGATCGCGCCTGGGCCGATAGGTCCGGGTAATCTT

TGCAAATTTAATCGTGCTGGGGATAGATCATTGTAATTATTGATCTTCAACGAGGAATTC
CTAGTAAGCGCGAGTCATCAGCTCGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCC
CGTCGCTCCTCCCGATTGAATGGTCCGGAGAAATCCTGGGAGCCCGGCCTCTACTCAACC
CGCGCAAGGGGCCTGACTGGTTGATCCTGCCAGTAGTGATATGCTTGTCTCAAAGACTAA
GCCATGCAAGTTAAGAGAGATTTAGC

53 18S (*Acanthamoeba*)

GTAAATGTCTCAGACTAGCCATGCATGTGTAAGTATAAACTGTGTTATACAGGTGAGACC
TGGGGATAGGATCAACAAGTCAGTTATAGTTTATTTGATGGTCTCTTTTGTCTTTTTTTAC
CTACTTGGATAACCGTGGTAATTCTAGAGCTAATACATGCGCAAGGTCCCGAGCGCGGG
GGGCAGGGCTTCACGGCCCTGTCCTCGCATGCGCAGAGGGATGTATTTATTAGGTTAAAA
ACCAGCGGCAGGGGTCAGCAATGGCCCCTGCCAAACACTCCTGGTGATTCATAGTAACTC
TTTCGGATCGCATTCATGTCCTCCTTGTGGGGACGGCGACGATTCATTCAAATTTCTGCCC
TATCAACTTTCGATGGTAGGATAGAGGCCTACCATGGTCGTAACGGGTAACGGAGAATT
AGGGTTCGATTCCGGAGAGGGAGCCTGAGAAATGGCTACCACTTCTAAGGAAGGCAGCA
GGCGCGCAAATTACCCAATCCCGACACGGGGAGGTAGTGACAATAAATACAATACAGGC
GCTCGATAAGAGTCTTGTAATTGGAATGAGTACAATTTAAACCCCTTAACGAGTAACAAT
TGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAA
AGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATCTAGGGACGCGCATTTCAAGCGCCCGTG
TCGTCGGGTCAAACCGGCGACTGCGTTGGCGTTGCGGGCTCGGTCCGTCGGTGGACCCTC
GTGGTCTTAATCGGCGTGTCAACCGGCCCGCCCGTCCCCTCCTTCTGGATTCCCGTTCCTG
CTATTGAGTTAGTGGGGACGTCACAGGGGGCTCATCGTCGTCATGCAAATGGCGGCGGT
GGGTCCCTGGGGCCCAGATCGTTTACCGTGAAAAAATTAGAGTGTTCAAAGCAGGCAGA
TCCAATTTTCTGCCACCGAATACATTAGCATGGGATAATGGAATAGGACCCTGTCCTCCT
ATTTTCAGTTGGTTTTGGCAGCGCGAGGACTAGGGTAATGATTAATAGGGATAGTTGGGG
GCATTAATATTTAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGATTAACTGCCAAG
GATGTTTTCATAAATCAGGAACGAAAAGTAGGGGATCGAAGACGATCAGATACCGTCGT
AGTCTTAACCCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAACACCA
CCATCGGCGCGGTCGTCCTTGGCGTCTGTCCCTTTCAACGGGGGCAGGCGCGAGGGCGGT
TTAGCCCCGGTGGCACCGGTGAATGACTCCCCTAGCAGCTTGTGAGAAATCATAAGTCTT
TGGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGGAATTGACGGAAGGGCA
CCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTTACCAGGTCC
GGACATAGTAAGGATTGACAGATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCAT
GGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCTTA
ACCTGCTAAATATGCCGCGCTAACCCGTCCATCAAAACCCATGCGTGGCTCACGCGGTCC
GCTGCGGGGTGGTGTCGCTTCGCGGCGACGTCATCCCGCCGGCAGGGCCCGGGTCCGTGT
GGGCGGTAGGGTTCGGCGTCCGTGCTTCTTAGAGGGACTGCTGCGCGCCTAGCCAGCGG
AAGTTTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTA

CACTGATTAATCCAACGAGTCCGCTTCAATCGAGGCGCGATGCCGTTGGGGTCAAACCCA
ACTGTGTCGCTGTCCTCGATCGCGCCTGGGCCGATAGGTCCGGGTAATCTTTGCAAATTT
AATCGTGCTGGGGATAGATCATTGTAATTATTGATCTTCAACGAGGAATTCCTAGTAAGC
GCGAGTCATCAGCTCGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCT
ACCGATTGAATGGTCCGGTGAAATCCTCGGAGCCGTGGCCTCTACGCAATCCGGGCAACC
GGGTGAGAGGTTTATCCCTTTCAGCTAGTGAAATGCGTTGTCTACACAAGCCTAAGAGCA
GACAGGAGATAGTGATAAAAAGCCCCGC

60 18S (*Acanthamoeba*)

AGCCATGCATGTCTAAGTATAAGCTTGTTTATACGGCGAGACTGCGGATGGCTCATTAAA
TCAGTTATAGTTTATTTGATGGTCTCTTTGTTCTTCTTTTTACCTACTTGGATAACCGTGGT
AATTCTAGAGCTAATACATGCGCAAGGTCCCGAGCGCGGAGGGTAGGGCTTCACGGCTC
TGTCCTTGCATGCGCAGAGGGATGTATTTATTAGGTTAAACAACCAGCATTTTCATATCA
TCTGGGGATTCATAGTAACTCTTTCGGATCGCATTCTTGCCCTCCTTGTGGGGGCGGCGAC
AATACATTCTCATTTCTGCCCTATCAACTTTCGATGGGGGGATAGAGGCCTACCCTGGGC
GTAACGGGTAACGGAGAATTATGGTTCGATTCCCGAGAGGGAGCCTGAGAAATGGGTAC
CACTTCTAAGGGAAGCAGCGGGCGCACAAATCACTCTATCCCACACGGGGGGAGGTAGA
GACAATAAATAACAATACACGCGCTAGAAAAGAGTCTTGTAATAGGAGTGAGCACTATT
TACCCCCCTTAGCGAGAAACAATGGGAGGGCATGTCTGGTGCAAGCACGCGCGGTAATT
CCAGCTCCAAGAGTATATAAGAAAGTTGTCGCAGAAAAATAGCTTGTAGTTGTATCTAGG
GGCGCGCATTTATTTAAGCATGCGTGTCATCGTGTCAAACCGATGACTGCGTGGTGTTGC
GGGCTCTGTACATTTCTCATCCCACAAGGGTGAATAGTGTGTTAACCGCCCCCCCTGTCC
CCTCTTTGGGGATTCCCGCTCGTGATATTGAGTTAGTGGGGACGTCACAGGGATCTTATC
ATTGGTTTCACGCCCAGTGGTAAGTTTGTGGGGCCCAGATTGTTTCCCGTGAAAAAATTA
GAGTGTTCAAAGCAGGCAGATTCATTTTCTGCCACCGAATACATTAGCATGGGATAATGG
AATAGGACCCTGTCCTCCTATCTTCAGTTGGTTAACTTGTAGAGGATCAGGGTAATGATT
AATAGGGATAGTTGGGGGCATTAATATTTAATTGTCAGAGGTGAAATTCTTGGATTTATG
AAAGATTAACTTCTGCGAAAGCATCTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGT
TAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCATAAACGATGCCGACCAG
CGATTAGGAGACGTTGAATACAACACACCCCCATATATATTCAGTACATGCCAGTCAAGT
GGTTGTGTATTGTGTGTGTAGGCACCGGTGAATGACTCCCCTAGCAGCTTGTGAGAAATC
ATAAGTCTTTGGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGGAATTGAC
GGAAGGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTT
ACCAGGTCCGGACATAGTAAGGATTGACAGATTGATAGCTCTTTCTTGATTCTATGGGTG
GTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGTTAACGAAC
GAGACCTTAACCTGCTAAATATGCCGCGCTAACCCGTCCATCAAAACCCATATGTGACTC
ACGCGGTTCGCTGCAAAGTAATGCTGTCAGTCAAATGATGGTGTTGATTTGCCGGCAGGG
CCCGGGTTCATGTGGGCGGTAGGGTTCGGCGTCTGTGCTTCTTAGAGGGACTGCTGCGCG

CCTAGCCAGCGGAAGTTTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCC
GCACGCGCGCTACACTGATTAATCCAACGAGTCCGCTTCAATCGAGGCGTAGTATATGTG
GGGTCAAACCTATGTATGTTGCTGTCCTTGATCGCGCCTGGGCCGATAGGTCCGGGTAAT
CTTTGCAAATTTAATCGTGCTGGGGATAGATCATTGTAATTATTGATCTTCAACGAGGAA
TTCCTAGTAAGCGCGAGTCATCAGCTCGCGTTGATTACGTCCCTGCCCTTTGTACACACCG
CCCGTCGCTCCTACCGATTGAATGGTCCGGTGAAATCCTCGGAGCCGTGGCCTCTACGCA
ATCTGGGCAACCGGGTTGTGAGGTCAACGGTGGTGTGCAAGCATCACCCTATTCGGCGGC
GAAGTCGATTGAACACTTACCTATTAGACGACAGGAGAAGTCGATCATCCTGGG

61 18S (*Acanthamoeba*)

GGGGGTTTTAAGAAACTACTTTAGATAGCATGCATGTCTAGTATAAGCTTGTTTATACGG
CGAGACTGCGGATGGCTCATTAAATCAGTTATAGTTTATTTGATGGTCTCTTTGTTCTTCT
TTTTACCTACTTGGATAACCGTGGTAATTCTAGAGCTAATACATGCGCAAGGTCCCGAGC
GCGGAGGGTAGGGCTTCACGGTTCTATCCTTGCATGCGCAGAGGGATGTATTTATTAGGT
TAAACAACCAGCATTTTAACAATCAATCTGGTGATTCATAGTAACTCTTTCGGATCGCAT
TCATGCCCTCCTTGTGGGGGCGGCGACGATTCATTCAAATTTCTGCCCTATCAACTTTCGA
TGGTAGGATAGAGGCCTACCATGGTCGTAACGGGTAACGGAGAATTAGGGTTCGATTCC
GGAGAGGGAGCCTGAGAAATGGCTACCACTTCTAAGGAAGGCAGCAGGCGCGCAAATTA
CCCAATCCCGACACGGGGAGGTAGTGACAATAAATAACAATACAGGCGCTCGATAAGAG
TCTTGTAATTGGAATGAGTACAATTTAAACCCCTTAACGAGTAACAATTGGAGGGCAAGT
CTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTT
AAAAAGCTCGTAGTTGGATCTAGGGACGCGCATTTATTTAAGCATCCGTGTCATTGGGTC
AAACTGGTGACTGCGTGGTGTTGCGGGCTCGGTCCATTAGTCATCCCACAAGGGTGATTA
GTGTGTTAACCGGCCCGCCCGTCCCCTCCTTCTGGATTCCCGTTCCTGCTATTGAGTTAGT
GGGGACGTCACAGGGAGCTGTATCATTGGCCTTCACGGGCCAGTGGTACAGTTTCTGGGG
CCCAGATCGTTTACCGTGAAAAAATTAGAGTGTTCAAAGCAGGCAGATTCAATTTTCTGC
CACCGAATACATTAGCATGGGATAATGGAATAGGACCCTGTCCTCCTATCTTCAGTTGGT
TAACTTGTAGAGGATCAGGGTAATGATTAATAGGGATAGTTGGGGGCATTAATATTTAAT
TGTCAGAGGTGAAATTCTTGGATTTATGAAAGATTAACTTCTGCGAAAGCATCTGCCAAG
GATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGT
AGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAACACCAC
CATACATAATTCAGTACATGGCCAGTCAAATGGTTGTGTATTGTTTGTGTATGGCACCGG
TGAATGACTCCCCTAGCAGCTTGTGAGAAATCATAAGTCTTTGGGTCCGGGGGGAGTATG
TCGCATGATCCCT

63 18S (*Acanthamoeba*)

GTCTGTTAGACTAGCCATGCATGTGTAAGTATAAAGCTGCATTATACAGGCTGGAGACCT
TGCCGATGGCATCAATAAATCAAGTTATAGTTTATTTGATGGTCTCTTTTGTCTTTTTTTAC

CTACTTGGATAACCGTGGTAATTCTAGAGCTAATACATGCGCAAGGTCCCGAGCGCGGG
GGGCGGGGCTTCACGGCCTCGTCCTCGCATGCGCAGAGGGATGTATTTATTAGGTTAAAA
ACCAGCAGCCGGCAACGGCTTCAACTCCTGGTGATTCATAGTAACTCTTTCGGATCGCAT
TCATGTCCTCCTTGTGGGGACGGCGACGATTCATTCAAATTTCTGCCCTATCAACTTTCGA
TGGTAGGATAGAGGCCTACCATGGTCGTAACGGGTAACGGAGAATTAGGGTTCGATTCC
GGAGAGGGAGCCTGAGAAATGGCTACCACTTCTAAGGAAGGCAGCAGGCGCGCAAATTA
CCCAATCCCGACACGGGGAGGTAGTGACAATAAATAACAATACAGGCGCTCGATAAGAG
TCTTGTAATTGGAATGAGTACAATTTAAACCCCTTAACGAGTAACAATTGGAGGGCAAGT
CTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTT
AAAAAGCTCGTAGTTGGATCTAGGGACGCGCATTTCAAGCGCCCGTGCCATCGGGTCAA
ACCGGTGGCTGCGTTGGCGTTGCGGGCTCGGTCCGTCGGTGCCCCACAAAGGGCTATCGG
CGTGTCAACCGGCCCGCCCGTCCCCTCCTTCTGGATTCCCGTTCCTGCTATTGAGTTAGTG
GGGACGTCACAGGGGGTCCATCGTCGTGCGGCGTCAAAACCGTGCGGCGGTGGGTCCCT
GGGGCCCAGATCGTTTACCGTGAAAAATTAGAGTGTTCAAAGCAGCAGATCCAATTTTCT
GCCACCGAATACATTAGCATGGGATAATGGAATAGGACCCTGTCCTCCTATTTTCAGTTG
GTTTTGGCAGCGCGAGGACTAGGGTAATGATTAATAGGGATAGTTGGGGGCATTAATATT
TAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGATTAACTTCTGCGAAAGCATCTGC
CAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCG
TCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAACA
CCACCATCGGTGCGGTCGTCCTTGGCGTCTCGGTTCCTCCCGGGGTGCGGGGACGGGTTA
GGCTGATGGCGCCGGTGAATGACTCCCCTAGCAGCTTGTGAGAAATCATAAGTCTTTGGG
TTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCAC
CAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTTACCAGGTCCGGAC
ATAGTAAGGATTGACAGATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCC
GTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCTTAACCTG
CTAAATATGCCGCGCTAACCCGTCCATCAAAACCCATGCGCGGCTCACGCGGTCCGCTGC
GGGGTGGTGTCGCTTCGCGGCGACGTCATCCCGCCGGCAGGGCCCGGGTCCGTGTGGGC
GGTAGGGTTCGGCGTCCGTGCTTCTTAGAGGGACTGCTGCGCGCCTAGCCAGCGGAAGTT
TGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTG
ATTAATCCAACGAGTCCGCTTCAATCGAGGCGCGATGCCGTTGGGGTCAAACCCAACTGC
GTCGCTGTCCTCGATCGCGCCTGGGCCGATAGGTCCGGGTAATCTTTGCAAATTTAATCG
TGCTGGGGATAGATCATTGTAATTATTGATCTTCAACGAGGAATTCCTAGTAAGCGCGAG
TCATCAGCTCGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGA
TTGAATGGTCCGGTGAAATCCTCGGAGCCGTGGCCTCTACGCAATCCGGGCAACCGGGTG
AGAGGTCTCTCCCTTTCGGCGGCGAAGTGGAGTGAACACAAGACCTAAGCCAGACAGAG
AAGTCTAATACCGC

64 18S (*Acanthamoeba*)

GTCTGACTAGACTAGCCATGCATGTCTAAGTATAAGCTTGTTTATACGGCGAGACTGCGG
ATGGCTCAATAAATCAGTTATAGTTTATTTGATGGTCTCTTTTGTCTTTTTTTACCTACTTG
GATAACCGTGGTAATTCTAGAGCTAATACATGCGCAAGGTCCCGAGCGCGGGGGGCGGG
GCTTCACGGCCTCGTCCTCGCATGCGCAGAGGGATGTATTTATTAGGTTAAAAACCAGCA
GCCGGCAACGGCTTCAACTCCTGGTGATTCATAGTAACTCTTTCGGATCGCATTCATGTCC
TCCTTGTGGGGACGGCGACGATTCATTCAAATTTCTGCCCTATCAACTTTCGATGGTAGG
ATAGAGGCCTACCATGGTCGTAACGGGTAACGGAGAATTAGGGTTCGATTCCGGAGAGG
GAGCCTGAGAAATGGCTACCACTTCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATC
CCGACACGGGGAGGTAGTGACAATAAATAACAATACAGGCGCTCGATAAGAGTCTTGTA
ATTGGAATGAGTACAATTTAAACCCCTTAACGAGTAACAATTGGAGGGCAAGTCTGGTG
CCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTAAAAAG
CTCGTAGTTGGATCTAGGGACGCGCATTTCAAGCGCCCGTGCCATCGGGTCAAACCGGTG
GCTGCGTTGGCGTTGCGGGCTCGGTCCGTCGGTGCCCCACAAAGGGCTATCGGCGTGTCA
ACCGGCCCGCCCGTCCCCTCCTTCTGGATTCCCGTTCCTGCTATTGAGTTAGTGGGGACGT
CACAGGGGGTCCATCGTCGTGCGGCGTCAAAACCGTGCGGCGGTGGGTCCCTGGGGCCC
AGATCGTTTACCGTGAAAAATTAGAGTGTTCAAGCAGGCAGATCCAATTTTCTGCCACCG
AATACATTAGCATGGGATAAGGATAATGGAATAGGACCCTGTCCTCCTATTTTCAGTTGG
TTTTGGCAGCGCGAGGACTAGGGTAATGATTAATAGGGATAGTTGGGGGCATTAATATTT
AATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGATTAACTTCTGCGAAAGCATCTGCC
AAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGT
CGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAACAC
CACCATCGGTGCGGTCGTCCTTGGCGTCTCGGTCCTTCCCGGGGCGCGGGCACGGGGGAG
GCTTATGCCGGTGGCGACTGAGACCGACACCCCCATGAGCTAGTGAGAAATCATAAGTC
TTTGGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTAAAGGAATTGACGGAAGGGC
ACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTTACCAGGTC
CGGACATAGTAAGGATTGACAGATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCA
TGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCTTA
ACCTGCTAAATATGCCGCGCTAACCCGTCCATCAAAACCCATGCGCGGCTCACGCGGTCC
GCTGCGGGGTGGTGTCGCTTCGCGGCGACGTCATCCCGCCGGCAGGGCCCGGGTCCGTGT
GGGCGGTAGGGTTCGGCGTCCGTGCTTCTTAGAGGGACTGCTGCGCGCCTAGCCAGCGG
AAGTTTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTA
CACTGATTAATCCAACGAGTCCGCTTCAATCGAGGCGCGATGCCGTTGGGGTCAAACCCA
ACTGCGTCGCTGTCCTCGATCGCGCCTGGGCCGATAGGTCCGGGTAATCTTTGCAAATTT
AATCGTGCTGGGGATAGATCATTGTAATTATTGATCTTCAACGAGGAATTCCTAGTAAGC
GCGAGTCATCAGCTCGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCT
ACCGATTGAATGGTCCGGTGAAATCCTCGGAGCCGTGGCCTCTACGCAATCCGGGCAACC

GGGTGTGAGGTCTCCCCTTTCGGCGGCGAAGTCGAGTGAACCTTACCAAGAGACGAAGA
GAAGTCTAATACCGA

G5 18S (*Acanthamoeba*)

AGACTAGCCATGCATGTGTAAGTATAAGCTGGTTTATACGGGTGAGACTGCGGATGGCTC
AACACAATCAGTTATAGTTTATTTGATGGTCTCTTTTGTCTTTTTTTACCTACTTGGATAAC
CGTGGTAATTCTAGAGCTAATACATGCGCAAGGTCCCGAGCGCGGGGGGCAGGGCTTCA
CGGCCCTGTCCTCGCATGCGCAGAGGGATGTATTTATTAGGTTAAAAACCAGCGGCAGG
GGTCAGCAATGGCCCCTGCCAAACACTCCTGGTGATTCATAGTAACTCTTTCGGATCGCA
TTCATGTCCTCCTTGTGGGGACGGCGACGATTCATTCAAATTTCTGCCCTATCAACTTTCG
ATGGTAGGATAGAGGCCTACCATGGTCGTAACGGGTAACGGAGAATTAGGGTTCGATTC
CGGAGAGGGAGCCTGAGAAATGGCTACCACTTCTAAGGAAGGCAGCAGGCGCGCAAATT
ACCCAATCCCGACACGGGGAGGTAGTGACAATAAATAACAATACAGGCGCTCGATAAGA
GTCTTGTAATTGGAATGAGTACAATTTAAACCCCTTAACGAGTAACAATTGGAGGGCAAG
TCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGT
TAAAAAGCTCGTAGTTGGATCTAGGGACGCGCATTTCAAGCGCCCGTGTCGTCGGGTCAA
ACCGGCGACTGCGTTGGCGTTGCGGGCTCGGTCCGTCGGTGGACCCTCGTGGTCTTAATC
GGCGTGTCAACCGGCCCGCCCGTCCCCTCCTTCTGGATTCCCGTTCCTGCTATTGAGTTAG
TGGGGACGTCACAGGGGGCTCATCGTCGTCATGCAATGGCGGCGGTGGGTCCCTGGGGC
CCAGATCGTTTACCGTGAAAAATTAGAGTGTCAAAGCAGGCAGATCCAATTTCTGCCACC
GATACATTAGCATGGGATATGAATAGACCCTGTCTCTATTTCAGTGTTTTGCAGCGCGAG
GACTAGGTATGATTATAGGATAGTGGGGGCATAATATTAATTGTCAGAGTGAAATCTTGA
TTATGAAAGATACTTCTGCAAGCATCTGCAGATGTTGAAAGCATTCTGCCAAGGATGTTT
CATTAATCAAGACCGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAA
CCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATACAAAACACCACCATCGGCG
CGGTCGTCCTTGGCGTCTGTCCCTTTCAACGGGGGCAGGCGCGAGGGCGGTTTAGCCCGG
TGGCACCGGTGAATGACTCCCCTAGCAGCTTGTGAGAAATCATAAGTCTTGGGTTCCGGG
GGGAGTATGGTCGCAAGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGT
GGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTTACCAGGTCCGGACATAGTAA
GGATTGACAGATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAG
TTGGTGGAGTGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCTTAACCTGCTAAATA
TGCCGCGCTAACCCGTCCATCAAAACCCATGCGTGGCTCACGCGGTCCGCTGCGGGGTGG
TGTCGCTTCGCGGCGACGTCATCCCGCCGGCAGGGCCCGGGTCCGTGTGGGCGGTAGGGT
TCGGCGTCCGTGCTTCTTAGAGGGACTGCTGCGCGCCTAGCCAGCGGAAGTTTGAGGCAA
TAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGATTAATCC
AACGAGTCCGCTTCAATCGAGGCGCGATGCCGTTGGGGTCAAACCCAACTGTGTCGCTGT
CCTCGATCGCGCCTGGGCCGATAGGTCCGGGTAATCTTTGCAAATTTAATCGTGCTGGGG
ATAGATCATTGTAATTATTGATCTTCAACGAGGAATTCCTAGTAAGCGCGAGTCATCAGC

TCGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGATTGAATGG
TCCGGTGAAATCCTCGGAGCCGTGGCCTCTACGCAATCCGGGCAACCGGGTGAGAGGTC
TCTCCCTGTCTCGAGCGAGCGAAATGCGATGTGCTACACATAGCCTAATCCAGGACAGAG
AAGT

FF 18S (*Acanthamoeba*)

GGTCTGTCCAGATTAGCCATGCATGTCTAAGTATAAGCTTGTTTTATACGGCGAGACTGC
GGATGGCTCATTAAATCAGTTATAGTTTATTTGATGGTCTCTTGACACGGCGCAAGCCGT
ATCATTTACTTGGATAACTGTGGTAATTCTAGAGCTAATACATGCGTAAAATCTCGAGCG
CGGGGGGTGGTCTTGGGGTGCAGGTGGCAACACTTGTGCTCCGTTCGGGCCATCTTTGCA
TGCGCGAAGGGATGTATTTATTAGACACACAAACCAGCATTCTTTTTACTGGTGATTCAT
AGTAACTCTTTCGGATCGCATTTATATCTTCCTTGTGGAGATGGCGACGATTCATTCAAAT
TTCTGCCCTATCAACTTTCGATGGTAGGATAGAGGCCTACCATGGTCGTAACGGGTAACG
GAGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAATGGCTACCACTTCTAAGGAA
GGCAGCAGGCGCGCAAATTACCCAATCCCGACACGGGGAGGTAGTGACAATAAATAACA
ATACATGCGCTCGACAAGAGTCGTGTAATTGGAATGAGTACAATTTAAACCCCTTAACGA
GTAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCG
TATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTTGGGACGTGCATTCACAT
TCATAAAGGTGTAAAAGCCTGTGGAAGCGTCTTACTGCTCTATTGGCGCTAGGTCACCTT
CACGGGTGGTCTGGTGTCAAGGTAGTTTGGCGTTGCGGGCTCGGTCCATCAGTGTGCCTC
GCAAGGGGCCTCCATTGGTGTGTTAACCGGCCCGCCCGTCCCCTCCTTCTGGATTCCCGTT
CCTGCTATTGATTTAGTGGGAACGTCACAGGGGGCTAGTCGTGTAAAAAGCGGCTGGTCT
CTGGGGCCCAGATCGTTTACCGTGAAAAAATTAGAGTGTTCAAAGCGGGCAGATATTTTT
CCTGCCACCGAATACATTAGCATGGGATAATGGAATAGGACCCTGACCTCCTATTTTCAG
TTGGTTTTGTTTACAGCGAGGTTATATCAGGGTAATGATTAATAGGGATAGTTGGGGGCA
TTAATATTTAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGATTAACTTCTGCGAAA
GCGTTTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATC
AGATACCGTCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAAT
ACAAAACACCGCTGTTAATCCTTTCGGGGGTTAATAGTTGGTGAATGACTCCCCTAGCAG
CTTGTGAGAAATCATAAGTCTTTGGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTT
AAAGGAATTGACGGAAGGGCACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAAC
ACGGGGAAACTTACCAGGTCCGGACATAGTGAGGATTGACAGATTGATAGCTCTTTCTTG
ATTCTATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTTTATTC
CGTTAACGAACGAGACCTTAGCCTGCTAAATAAGCCGCGCTAACCCATCCATCAAAACCC
ATGCGCGGCTTCCGAGGTCCGCTGCAAGGTGGCGTCGCTTCACGGCGGCTGTCATTTTGC
CGGTGGGATCCGAGCTCGTGTGGGCGGTCGGGTTCGGCGTCCGTGCTTCTTAGAGGGACT
GCTGATTATTTAAAGCGTTTAGCCAGCGGAAGTTTGAGGCAATAACAGGTCTGTGATGCC
CTTAGATGTTCTGGGCCGCACGCGCGCTACACTGATTAATCCAACGAGTCCGCTTCAATC

GGCACGTTCATGTGCGGTGGGTCAAACCATCGTATTGTGAGCTGTGTCGATCGCGCCTGG
GCCGATAGGTCCGGGTAATCTTTGCAAATTTAATCGTGCTGGGGATAGATCATTGTAATT
ATTGATCTTCAACGAGGAATTCCTAGTAAGCGCGAGTCATCAGCTCGCGTTGATTACGTC
CCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGATTGAATGGTCCGGTGAAATCCTC
GGAGCCATGGCCTCTACGCAATCCGGGCAACCGGGTTGTGAGGTTTCGCCGCAAGGCGA
TTCTGGTGGCGAAGTCGATTGAACCTTACCATTAGAGAAGAGAAGTCGACACTCCGG

BC 18S (*Acanthamoeba*)

TGGCTTCTGTCTCAGATTAGCCATGCATGTCTAAGTATAAGCTTGTTTTATACGGCGAGAC
TGCGGATGGCTCATTAAATCAGTTATAGTTTATTTGATGGTCTCTTGACACGGCGCAAGC
CGTATCATTTACTTGGATAACTGTGGTAATTCTAGAGCTAATACATGCGTAAAATCTCGA
GCGCGGGGGGTGGTCTTGGGGTGCAGGTGGCAACACTTGTGCTCCGTTCGGGCCATCTTT
GCATGCGCGAAGGGATGTATTTATTAGACACACAAACCAGCATTCTTTTTACTGGTGATT
CATAGTAACTCTTTCGGATCGCATTTATATCTTCCTTGTGGAGATGGCGACGATTCATTCA
AATTTCTGCCCTATCAACTTTCGATGGTAGGATAGAGGCCTACCATGGTCGTAACGGGTA
ACGGAGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAATGGCTACCACTTCTAAG
GAAGGCAGCAGGCGCGCAAATTACCCAATCCCGACACGGGGAGGTAGTGACAATAAATA
ACAATACATGCGCTCGACAAGAGTCGTGTAATTGGAATGAGTACAATTTAAACCCCTTAA
CGAGTAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATA
GCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGGATTTTGGGACGTGCATTCA
CATTCATAAAGGTGTAAAAGCCTGTGGAAGCGTCTTACTGCTCTATTGGCGCTAGGTCAC
CTTCACGGGTGGTCTGGTGTCAAGGTAGTTTGGCGTTGCGGGCTCGGTCCATCAGTGTGC
CTCGCAAGGGGCCTCCATTGGTGTGTTAACCGGCCCGCCCGTCCCCTCCTTCTGGATTCCC
GTTCCTGCTATTGATTTAGTGGGAACGTCACAGGGGCTAGTCGTGTAAAAAGCGGCTGGT
CTCTGGGGCCCAGATCGTTTACCGTGAAAAATTAGAGTGTCAAGCGGCAGATATTTTTTC
CTGCCACCGAATACATAGCATGGATATGGATAGGACCCTGACTCATTTCAGTGATTTGTT
TACAGCGAAGTAATTCAGGTATGAATATAGGGATAGGTCGGGGCATAATATTATTGGTC
AGAGTGAAATCTGATTATGAAAGATACTTCTGCGAAGCGTTTGCAAGGATGTTTTCATAT
CAGACGAAAGTTAGGGATCGAAGACGATCAGATACCGTCGTAGTCTACCCATAAACGAT
GCCGACCAGCGATTAGGAGACGTTGAATACAAACACCGCTGTTAATCCTTTCGGGGGTTA
ATAGTTGGTGAATGACTCCCCTAGCAGCTTGTGAGAAATCATAAGTCTTTGGGTTCCGGG
GGGAGTATGGTCGCAAGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGAGT
GGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTTACCAGGTCCGGACATAGTGA
GGATTGACAGATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCATGGCCGTTCTTAG
TTGGTGGAGCGATTTGTCTGGTTTATTCCGTTAACGAACGAGACCTTAGCCTGCTAAATA
AGCCGCGCTAACCCATCCATCAAAACCCATGCGCGGCTTCCGAGGTCCGCTGCAAGGTG
GCGTCGCTTCACGGCGGCTGTCATTTTGCCGGTGGGATCCGAGCTCGTGTGGGCGGTCGG
GTTCGGCGTCCGTGCTTCTTAGAGGGACTGCTGATTATTTAAAGCGTTTAGCCAGCGGAA

GTTTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACA
CTGATTAATCCAACGAGTCCGCTTCAATCGGCACGTTCATGTGCGGTGGGTCAAACCATC
GTATTGTGAGCTGTGTCGATCGCGCCTGGGCCGATAGGTCCGGGTAATCTTTGCAAATTT
AATCGTGCTGGGGATAGATCATTGTAATTATTGATCTTCAACGAGGAATTCCTAGTAAGC
GCGAGTCATCAGCTCGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCT
ACCGATTGAATGGTCCGGTGAAATCCTCGGAGCCATGGCCTCTACGCAATCCGGGCAACC
GGGTTGTGAGGTCTCGCCGCAAGGCGATTTGGTGGCGAAGTCGATTGAACCTTACCATTA
GAGGAAGGAGAAGTCGAGCAAGATCGGGC

NSS 18S (*Acanthamoeba*)

CGGAATGTCTCAGACTAAGCCATGCAGTGTAAGTATAAACTGCATTATACAGGTGAACCT
GCGAAAGGATCAACCAGTCAGGGTAGTTTATTTGATGGTCTCTTTTGTCTTTTTTTACCTA
CTTGGATAACCGTGGGAATTCTAGAGCTAATACATGCGCAAGGTCCCGAGCGCGGGGGG
CGGGGCTTCACGGCCCCGTCCTCGCATGCGCAGAGGGATGTATTTATTAGGTTAAAAACC
AGCAGCCGGCAACGGCTTCAACACTCCTGGTGATTCATAGTAACTCTTTCGGATCGCATT
CATGCCCTCCTTGTGGGGGCGGCGACGATTCATTCAAATTTCTGCCCTATCAACTTTCGAT
GGTAGGATAGAGGCCTACCATGGTCGTAACGGGTAACGGAGAATTAGGGTTCGATTCCG
GAGAGGGAGCCTGAGAAATGGCTACCACTTCTAAGGAAGGCAGCAGGCGCGCAAATTAC
CCAATCCCGACACGGGGAGGTAGTGACAATAAATAACAATACAGGCGCTCGATAAGAGT
CTTGTAATTGGAATGAGTACAATTTAAACCCCTTAACGAGTAACAATTGGAGGGCAAGTC
TGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGCAGTTA
AAAAGCTCGTAGTTGGATCTAGGGACGCGCATTTCAAGCGCCCGTGCCGTCGGGTCAAA
CCCGGCGGCTGCGTTGGCGTTGCGGGCCTCGGGTCCGTCGGTGCCCCACAAAGGGCTATC
GGCGTGTCAACCGGCCCCGCCCCGTCCCCTCCTTCTGGATTCCCGTTTCCTGCTATTGAGT
TAGTGGGGACGTCACAGGGGGCTCATCGTCGTCGTGCCAAAACACGGCGGCGGTGGGTC
CTGTGGGCCCAGATCGTTTACCGTGAAAAAATTAGAGTGGTTCAAGCAGGCAGATCCAA
TTTTCTGCCACCGAATACATTAGCATGGGATAATGGAATAGGACCCTGTCCTCCTATTTTC
AGTTGGTTTTGGCAGCGCGAGGACTAGGGTAATGATTAATAGGGATAGTTGGGGGCATT
AATATTTAATTGTCAGAGGTGAAATTCTTGGATTTATGAAAGATTAACTTCTGCGAAAGC
ATCTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATCGAAGACGATCAG
ATACCGTCGTAGTCTTAACCATAAACGATGCCGACCAGCGATTAGGAGACGTTGAATAC
AAAACAACCACCATCGGTGCGGTCGTCCTTGGCGTCGGTTTCGGCCGGCGCGGGGGCGG
CTTAGCCCGGTGGCACCGGTGAATGACTCCCCTAGCAGCTTGTGAGAAATCATAAGTCTT
TGGGTTCCGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGGAATTGACGGAAGGGCA
CCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGGAAACTTACCAGGTCC
GGACATAGTAAGGATTGACAGATTGATAGCTCTTTCTTGATTCTATGGGTGGTGGTGCAT
GGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCTTA
ACCTGCTAAATATGCCGCGCTAACCCGTCCATCAAAACCCATGCGCGGCTCACGCGGTCC

GCTGCGGGGTGGTGTCGCTTCGCGGCGACGTCATCCCGCCGGCAGGGCCCGGGTCCGTGT
GGGCGGTAGGGTTCGGCGTCCGTGCTTCTTAGAGGGACTGCTGCGCGCCTAGCCAGCGG
AAGTTTGAGGCAATAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTA
CACTGATTAATCCAACGAGTCCGCTTCAATCGAGGCGCGGTGCCGTTGGGGTCAAACCCA
ACTGCGCGTCGCTGTCCTCGATCGCGCCTGGGCCGATAGGTCCGGGTAATCTTTGCAAAT
TTAATCGTGCTGGGGATAGATCATTGTAATTATTGATCTTCAACGAGGAATTCCTAGTAA
GCGCGAGTCATCAGCTCGCGTTGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTC
CTACCGATTGAATGGTCCGGTGAAATCCTCGGAGCCGTGGCCTCTACGCAATCCGGCCAA
CCAGGGGGACTGGTTGATCCTGCCAGTAGTGATATGCTTGTCTCAAAGACTAAGCCATGC
AAGTGTAAGTTAAGGATCGTC

Sheep 18S (*Vannella*)

GAGATTGAGGATACAAGACTAGCCATGCAAGTGTAGTATAAACTGCATTATACAGGTGA
AACTGCGAATGGCTCATTAAATCAGTTATAGTTTATTTGATGATTTTGGGTAGCAATACC
CACTACATGGATATCTGTAGAAACCTAGAGCTAATACATGCAAAATAACCAAACTTTTGG
GAATGGTTAGCACTTATTAGATACCAAACCAATATGGGGTCAAACCCATTTCAAGGTGAT
TCATAATAACTGAACAGATCGCATGGCCTTGTGCTGGCGACGTTTCATTCAAATTTCTGC
CCTATCAACTTGTTTTTAGTATAGAGGACTAAAAAGGTTTTTACGGGTAACGGAGAATTG
GGGTTCGATTCCGGAGAAGGAGCATGAGAAACGGCTACTATTTCTAAGGAAAGCAGCAG
GCGCGCAAATTATTCAATGAAAACAAGTTTTCGAAATAGTGACAAAAAATAACAAGGCG
GAAATTTTATTTTCGTGATTGGAATGAGTACAATTTAAATCCCTTAACAAGTAACAATTG
GAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAATAT
TGTTGCAGTTAAAACGCTCGTAGTTGAATATTGAGTATTGCATTGAATTGGTTGCACGTTT
GTGTAACTATTAATCATACTCTTCCTACTGGTAACTCGATTCTGTGCTTAATTGCATGGAG
TAGCATGATTTCCGGTTGTGTCGGTTGGTTATTTCAGCTTCCACAAAGAGGTTGAGAGGT
GTAAAGCCTTGAGATTGATTTAGTGGGTCAGGTTGAAGTGGCTATGGAGTTCTAGTCATT
TACTTTGAGAAAATTAGAGTGGTTAAGCAGCTTCTTTGGGTTCTGGATACATTAGCCATG
GGATAATAGAATAAGGATTTATCTGACATCTGTCGTTCACCAGTGAAATATGATGAACAG
GGACAGCTGGGGGATATTTATATTGGATGGTTAGCGGTGAAATGCTCGGATCCATTCAAG
ATAAACTAAAGCGAAAGCATTTACCAATGATTGTTTTCATTAATCAAGAACGAAAGTTAA
GGGATCGAAGACGATCAGATACCGTCGTAGTCATAAACTATAAACTATGCCGACCAGGG
ATGTGGGTTGTTATGAACATGTCAAAGTATTCGATTTCTTCCTATGGATCTTGTGAGAAAT
CATAAGTTTTTGGGTTCTGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGGAATTGAC
GGAGGGGCACCACCAGGAGTGGAGCATGCGGCTTAATTTGACTCAACACGGGGAAAACT
TACCAGGTCCAGACATTAGCAGGATTGACAGATTGATAGCTCTTTCATGATTTAATGGGT
GGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGATAACGAA
CGAGACCTTTACCATACTAAATAGTTTAATTAATGTTTACATTGACTCAAACTTCTTAGAG
GGACTTTTGACATCAAGTCAAAGGAAGCAAAGGCGATAACAGGTCTGTGATGCCCTTAG

ATGTTCTGGGCTGCACGCGTGCTACAATGATAAGCTCATCAAGTTGATTCGTTCATTCGA
ATAATGCTTCACCGGAAGGTGTGGCTAATCTTCAACGCTTATCGTGCTTGGGATAGATGC
TTGCAATTATTTATCTTGAACCAGGAATTCCTAGTAATCGTAATTCATCAGATTGCGATGA
TTACGTCCCTGCCCCTTGTACACACCGCCCGTCGCTGCTACCGATTGGATGATCCGGTGA
AATCCTCGGATTGTTGAAGATCCTTTTATACAAGGTAAATCTCTGACGAGAAATTGATTC
TACACTTATCATATCCACGACGCAATAGTTGAACCCCGAACCCCC

Cow1.2 18S (*Vannella*)

TTTGGAAGAAAGGACAAGATAAGCCATGCAGTGTAAGTATAAACTGCATTATACAGGTG
AAACTGCGAATGGATAAATAAATAAATTATAGTTTATTTGATGATTTTGGGTAGCAATAT
CTAATAAATGGATATCTGTAGAAACCTAGAGCTAATACATGCAAAATAACCAAACTTTTG
GGAATGGTTAGCACTCCCAAAGATACCAAACCAATATGGGGTCAAACCCATTTCAAGGT
GATTCATAATAACTGAACAGATCGCATGGCCTTGTGCTGGCGACGTTTCATTCAAATTTC
TGCCCTATCAACTTGTTTTTAGTATAGAGGACTAAAAAGGTTTTTACGGGTAACGGAGAA
TTGGGGTTCGATTCCGGATAAGGAGCATGAGAAACGGCTACTATTTCTAAGGAAAGCAG
CAGGCGCGCAAATTATTCAATGAAAACAATTTTCGAAATAGTGACAAAAAATAACAAGG
CGGAAATTTTATTTTCGTGATTGGAATGAGTACAATTTAAATCCCTTAACAAGTAACAAT
TGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAT
ATTGTTGCAGTTAAAACGCTCGTAGTTGAATATTGAGTATTGCATTAAATTGGTTGCACG
TTTGTGTAACTATTTAATCATACTCTTCCTACTGGTAACTCGATTCTGTGCTTAATTGCAT
GGAGTAGCATGATTTCGGTTGTGTCGGTTGGTTATTTCAGCTCCACAAAGAGGTTGAGAG
GTGTAAAAGCCTTGAGATTGATTTAGTGGGTCAAGTTGAAGTGGCTAATTGGAGTTCTAG
TTCATTTACTTTGAGAAAATTAGAGTGTTTAAAGCAGCCTCTTTGGGCTCTGAATACATTA
GCATGGGATAATAGAATAGGATTTTAATCTGACATTTGTTGGTTCAACAGGTTGAAATAA
TGATGAATAGGGACAGTTGGGGGTATTTATATTGGATGGTTAGCGGTGAAATGCTCGGAT
CCATTCAAGATAAACTAAAGCGAAAGCATTTACCAAGGATGTTTTCATTAATCAAGAACG
AAAGTTAAGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACTATAAACTATGCCG
ACCAGGGATGTGGGTTGTTATGAACATGTCAAAGCATTCGATTTCTTCCTATGGATCTTGT
GAGAAATCATAAGTTTTTGGGTTCTGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAG
GAATTGACGGAGGGGCACCACCAGGAGTGGAGCATGCGGCTTAATTTGACTCAACACGG
GGAAACTTACCAGGTCCAGACATTAGCAGGATTGACAGATTGATAGCTCTTTCATGATTT
AATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGA
TAACGAACGAGACCTTTACCATACTAAATAGTTTAGTTAATATTTATATTGATTCAAACTT
CTTAGAGGGACTTTTGACATCAAGTCAAAGGAAGCAAAGGCGATAACAGGTCTGTGATG
CCCTTAGATGTTTCTGGGCTGCACGCGTGCTACAAATGATAAAGCTCATCAAAGTTGATT
TCGTTCATTGGAATAATGCTTTCACCCGAAAGGTGTGGGCTAATCTTTCAACGCTTATCGT
GCTGGGGATAGATGCTGCAAATTATTATTCTGGACCCAGAAATTCCTAGTAATCGTAATT
CATCAGATTGCGATGATTACGTCCCTGCCCCTTGTACACACCCCCCGCGCTGCTACCCGA

TGGGATGATCCGGGGAAACCCTCGGATGATGGAGGTCCCTTTAATCCCAAGGTAAAAGT
CTGGAGAAGAAGGGTTTAAACCATCTCATAGAGAGAAGCATAAGCGAACGC

Cow2.1 18S (*Vannella*)

GGGGGAATGGGTCTCAGACTAAGCCATGCAGTGTAAGTATAAACTGCATTATACAGGTG
AAACTGCGAATGGCTCATTAAAACAGTTATAGTTTATTTGATGATTTTGGGTAGCAATAC
CCACTACATGGATATCTGTAGAAACCTAGAGCTAATACATGCAAAATAACCAAACTTTTG
GGAATGGTTAGCACTTATTAGATACCAAACCAATATGGGGTCAAACCCATTTCAAGGTGA
TTCATAATAACTGAACAGATCGCATGGCCTTGTGCTGGCGACGTTTCATTCAAATTTCTGC
CCTATCAACTTGTTTTTAGTATAGAGGACTAAAAAGGTTTTTACGGGTAACGGAGAATTG
GGGTTCGATTCCGGAGAAGGAGCATGAGAAACGGCTACTATTTCTAAGGAAAGCAGCAG
GCGCGCAAATTATTCAATGAAAACAAGTTTTCGAAATAGTGACAAAAAATAACAAGGCG
GAAATTTTATTTTCGTGATTGGAATGAGTACAATTTAAATCCCTTAACAAGTAACAATTG
GAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAATAT
TGTTGCAGTTAAAACGCTCGTAGTTGAATATTGAGTATTGCATTGAATTGGTTGCACGTTT
GTGTAACTATTCTAATCATACTCTTCCTACTGGTAACTCGATTCTGTGCTTAATTGCATGG
AGTAGCATGAATTTCGGTTGTGTCGGTTGTTATTTCAGCTCCACAAGAGGTGAGAGGTGT
AAAAGCCTTGAGATTGATTTAGTGGGTCAAGTTGAAGTGGCTAATTGGAGTCTAGTCTTT
ACTTTGAGAAATTAGAGTGTTTAAGCAACCTCTTTGGGCTCTGAATACATTAGCATGGGA
ATAATAGAAATAGGATTTATTCTGACATTTGTTGGGTTCACACGTGAAATATGATGAATA
CGGACAGTTGGGGTATTTATATTGGATGGTTTAGCGGTGAAATGCTGGGATCCATTCAAG
ATAAAGTAAAGTGAAAGCATTTACCATGGATGTTTCATTAATCCAAGAACGAAAGTTAA
GGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACTATAAAACTATGCCGACCAGGG
ATGTGGGTTGTTATGAACATGTCAAAGTACTCGATTTCATTCCTATGGATCTTGTGAGAA
CATCATAAGTTTTTTGGGTTCTGGGGGGAGTATGGTCGCAAGGCTGAAAATTAAAGGAAT
TGACGGAGGGGCACCACCAGGAGTGGAGCATGCGGCTTAATTTGACTCAACACGGGGAA
ACTTACCAGGTCCAGACATTAGCAGGATTGACAGATTGATAGCTCTTTCATGATTTAATG
GGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGATAAC
GAACGAGACCTTTACCATACTAAATAGTTTAATTAATATTTACATTGATTCAAACTTCTTA
GAGGGACTTTTGACATCAAGTCAAAGGAAGCAAAGGCGATAACAGGTCTGTGATGCCCT
TAGATGTTCTGGGCTGCACGCGTGCTACAATGATAAGCTCATCAAGTTGATTCGTTCATT
CGAATAATGCTTCACCGGAAGGTGTGGCTAATCTTCAACGCTTATCGTGCTTGGGATAGA
TGCTTGCAATTATTTATCTTGAACCAGGAATTCGTAGTAATCGTAATTCATCAGATTGCGA
TGATTACGTCCCTGCCCCTTGTACACACCGCCCGTCGCTGCTACCGATGGATGATCCGGT
GAAATCCTCGGATTGTTGAAGATCCTTTAATTACAAGTTAATACCTGCTCCACGTGATATT
CCTTCTCAAAGTCATATCGCAGACGC

Arn321 18S (*Vannella*)

CTAGACTAGCCATGCAGTGTAAGTATAAACTGCATTATACAGGTGAACCTGCGAATAGG
ATCAATCCAGTCAGTTATAGTTTATTTGATGATTTTGGGTAGCAATACCCACTACATGGAT
ATCTGTAGAAACCTAGAGCTAATACATGCAAAATAACCAAACTTTTCGAGAATGGTTAGC
ACTTATTAGATACCAAACCAATATGGGGTTAAACCCATTTATAGGTGATTCATAATAACT
GAACAGATCGCATGGCCTTGTGCTGGCGACGTTTCATTCAAATTTCTGCCCTATCAACTTG
TTTTTAGTATAGAGGACTAAAAAGGTTTTTACGGGTAACGGAGAATTGGGGTTCGATTCC
GGAGAAGGAGCATGAGAAACGGCTACTATTTCTAAGGAAAGCAGCAGGAACGCAAATT
ATTCAATGAAAACAAGTTTTCGAAATAGTGACAAAAAATAACAAGGCGGAAATTTTATT
TTCGTGATTGGAATGAGTACAATTTAAATCCCTTAACAAGTAACAATTGGAGGGCAAGTC
TGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAATATTGTTGCAGTTA
AAACGCTCGTAGTTGAATATTGAGTATTGTATTAAATTGGTTGCACGTTTGTGTAACTATT
TAATCGTACTCTTCCTACTGGTAACTCGATTCTGTGCTTAATTGCATGGAGTAGCATGATC
TCGGTTGTGTCGGTTAGTTATTTCAGCTCCACAAAGAGGTTGAGAGGTGTAAAAGCCTTG
AGATTAATTTAGTGGGTCAAGTTGAAGTGATTAATTGGAGTTCTAGTTCATTTACTTTGAG
AAAATTAGAGTGTTTAAAGCAGCCTCTTTGGGCTCTGAATACATTAGCATGGGATAATAG
AATAGGATTTTAATCTGATATTTGTTGGTTCAACAGGTTGAAATAATGATGAATAGGGAC
AGTTGGGGGTATTTATATTGGATGGTTAGCGGTGAAATGCTCGGATCCATTCAAGATAAA
CTAAAGCGAAAGCATTTACCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAAGGGAT
CGAAGACGATCAGATACCGTCGTAGTCTTAACTATAAACTATGCCGACCAGGGATGTGG
GTTGTTATGAATATGTCAAAGCATTCGATTTCTTCCTATGGATCTTGTGAGAAATCATAAG
TTTTTGGGTTCTGGGGGGAGTATGGTCGCAAGGCTGAAACTTAAAGGAATTGACGGAGG
GGCACCACCAGGAGTGGAGCATGCGGCTTAATTTGACTCAACACGGGGAAACTTACCAG
GTCCAGACATTAGCAGGATTGACAGATTGATAGCTCTTTCATGATTTAATGGGTGGTGGT
GCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGATAACGAACGAGAC
CTTTACCATACTAAATAGTTTAGTTAATATTTATATGATTCAAACTTCTTAGAGGGACTTT
TGACATCAAGTCAAAGGAAGCAAAGGCGATAACAGGTCTGTGATGCCCTTAGATGTTTCT
GGGCTGCACGCGTGCTACAATGATAAGCTCATCAAGTTGATTCGTTTTATTGAATAATGC
TTCACCCGGAAGGGTGTGGCTAATCTTCAACGCTTATCGTGCTTGGGATAGATGCTTGCA
AATTATTTTTCTGGACCCAGGAATTCCTAGTAATCGTAATTCATCAGATTGCGATGATTAC
GTCCCTGCCCCTTGTACACACCCCCCGTCGCTGCTACCGATTGGATGATCCGGTGAAATC
CTCGGTTTGTGGAGGATCCTTTTATCCCAAGGAAAAATTTTTGAGGAGAAGTGGATTAAC

KC 18S (*Vannella*)

GCGATTTGTCCAGACTAGCCATGCAAGTGTAAGTATAGAACTGCATTATACAGTGTGAAC
CTGCAAGAGGATCAACCATGTCATTATAGTTTATTTGATGGATACGCTGTTCAGGCACTA
CATGGATAACTGTAGTAACATAGAGCTAATACATGCTTAAAGATCTGACCTTACGGAAG

GATTTGCACATATTAGATTTCAAACCAATATCCTTTCGGGGATTTTTTGGTGATTCATAAT
ATCTGAACAAATCGCATGGTCTTGTACCGGCAATGTATCAATCAAGTTTCTGCCCTATCA
ACTTGATGTTAGTGTATTGTACTAACATGGTGATAACGGGTGACAAAGGATTAGGGTTCG
ATCCTGGAAAGGGAGCATGAAAAACGGCTACCATTTCTAAGGAAAGCAGCAGGCGCGGA
AATTATTCAATGGTAATATCTTACCGAAATAGTGACAAAAAATACCGATGTTCTTCACTT
AGTGATGAACAATTGGAATGGACACAATCCAAACCTCTTAGTGAGTAACAATTAAAGGG
CAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCTAATAGCGTATATTAATACTGTTG
CAGTTAAAACGCTCGTAGTTGAACTTAGAATTATTACAAATTGTTTTACTTGATTGTAAA
ATGCTTGCTTAATTCTTATCTACTGAATACTCTACTCTGTACTTAATTGTATGGAGTATCG
CCTAGCCAGCGAAGTTGTGGTTAATTAACTCCTATCATTGTAACCAAGAGTAAAGTCTTG
AACGTTGTAGTCAATGGGGATCCAGGTTAATTGATTATATGGGGGTGGCCAGTTATTTAC
CTTTGAGTAAATTAGAGTGTTCAAAGCAGCTCTTGGGGCTTTGAATAGTTAGCATGGGAA
TAATTAGAAATAGGATTGGATTCCTATTTGTTGGTTTATGAATTCAATAATGATTTAAGA
GGGACAGTTGGGGGGCACTCATATTAAGTGGTTAGCGGTGAAATGTGTTGACCCACTTAA
GATGAACTAAAGCGAAAGCATTTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTA
AGGGATCGAAGACGATTAGATACCGTCGTAGTCTTAACTATAAACTATGCCGACCAGGG
ATATGAGTAGTTTATTTCTAAATCATTTAGATGATTCAGATACTTCTCCTTATGCACCTTG
TGAGAAATCATAAGTTTTTGGGTTCTGGGGGAAGTATGGTCGCAAGGCTGAAACTTAAA
GGAATTGACGGAGGGGCACCACCAGGAGTGGAGCGTGCGGCTTAATTTGACTCAACACG
GGAAAACTTACCAGGTCCAGACATTTGAAGGATTGACAGATTGATAGCTCTTTCATGATT
AAATGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCCG
ATAACGAACGAGACCTTTACCAGCTAAATAGTAATTATTAGGACTTGTCTTAGTAAGTTA
TACTTCTTAGAGGGACTATTGGTTTTTAAACCAAAGGAAGTAAAGGCGATAACAGGTCTG
TGATGCCCTTAGATGTTCTGGGCTGCACGCGCGCTACAATGATCAACTCATCAAGTTATC
TCAACTTGTTTGTGATTTACTTTTCCTGAAGGAATGGTTAATCTTCAACGTTGATCGTGCT
TGGGATAGATAATTGTAATTATTTATCTTGAACAAGGAATTCCTAGTAATCGTAATTCAT
CAGATTGCGATGATTACGTCCCTGCCCCTTGTACACACCGCCCGTCGCTGCTACCGATTG
AATAATCCGGTGAACTCGTCGGATTGTGGTAAATATTTATGACTGGTTGATCCTGCCAGT
AGTGATATGCTGTCTCACAGACTAAGCCATACGCGGTAGGTAAAAATTCCCGGG

LN 18S (*Vannella*)

GTGGTTTGTTAAGACTAAGCCATGCAGTGTTAGTATGAACTGCATTTATATAGTGGAAAC
TGCGAAAGGGGATCAACCATGCACATTATAGTTTATTTGATGGATACGCTGTTCAGGCAC
TACATGGATAACTGTAGTAACATAGAGCTAATACATGCTTAAAGATCTGACCTTACGGAA
GGATTTGCACATATTAGATTTCAAACCAATATCCTTTCGGGGATTTTTTGGTGATTCATAA
TATCTGAACAAATCGCATGGTCTTGTACCGGCGATGTATCAATCAAGTTTCTGCCCTATC
AACTTGATGTTAGTGTATTGTACTAACATGGTGATAACGGGGGACAGAGGATTAGGGTTC
GATCCTGGAGAGGGAGCATGAGAAACGGCTACCATTTCTAAGGAAAGCAGCAGGCGCGG

AAATTATTCAATGGTAATATCTTACCGAAATAGTGACAAAAAATACCGATGTTCTTCACT
TAGTGATGAACAATTGGAATGGACACAATCCAAACCTCTTAGTGAGTAACAATTAGAGG
GCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCTAATAGCGTATATTAATACTGTT
GCAGTTAAAACGCTCGTAGTTGAACTTAGAATTATTACAAGTTGTTTTACTTGATTGTAA
AATGCTTGCTTAATTCTTATCTACTGGATACTCTACTCTGTACTTAATTGTATGGAGTATC
GCCTAGCCAGCGGAGTTGTGGTTAATTAACTCCTATCATTGTATCAAGGTGTAAAAGCCT
GAAGTGCAGTCATGGGATCAAGTTAATTAATTATAATGGGGGTGCCAGTTAATTTACTTT
GAGTAATTTAGAGTGTTCAAAGCAGCCCTCTTGGGGGCTTTGAATAGTTTAGCATGGGAT
AATAAAAATAGGATTGGATTCTAATTTGGTGGTTTAATGAATCCAATAATGATTAAGAGG
GAACAGTTGGGGCACTCATATTAAGTGGTTAGCGGTGAAATGTGTTGACCCACTTAAGAT
GAACTAAAGCGAAAGCATTTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAAGG
GATCGAAGACGATTAGATACCGTCGTAGTCTTAACTATAAACTATGCCGACCAGGGATAT
GAGTAGTTTATTTCTAGATCATTTCGATGATTCAGATACTTCTCCTTATGCACCTTGTGAG
AAATCATAAGTTTTTGGGTTCTGGGGGAAGTATGGTCGCAAGGCTGAAACTTAAAGGAA
TTGACGGAGGGGCACCACCAGGAGTGGAGCGTGCGGCTTAATTTGACTCAACACGGGAA
AACTTACCAGGTCCAGACATTTGAAGGATTGACAGATTGATAGCTCTTTCATGATTAAAT
GGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAATTCCGATAA
CGAACGAGACCTTTACCAGCTAAATAGTAATTATTAAGACTTATCTTAGTAAGTTATACT
TCTTAGAGGGACTATTGGTTTTTAAACCAAAGGAAGTAAAGGCGATAACAGGTCTGTGAT
GCCCTTAGATGTTCTGGGCTGCACGCGCGCTACAATGATCAACTCATCAAGTTATCTCAA
CTTGTTTGTGATTTACTTTTCCTGAAGGAATGGTTAATCTTCAACGTTGATCGTGCTTGGG
ATAGATAATTGTAATTATTTATCTTGAACAAGGAATTCCTAGTAATCGTAATTCATCAGA
TTGCGATGATTACGTCCCTGCCCCTTGTACACACCGCCCGTCGCTGCTACCGATTGAATA
ATCCGGTGAACTCGTCGGATTGTGGTAAATATTTAGTGTTTGGGGTTCCCCCCCTAGTGAT
ATTGAGTCTCACAGACTAAGCCATCCGCGAATAATTTGTACAGCTGGGG

N**SS COI (*Acanthamoeba*)**

ATTATACTTAATATTTGGTGGGTTTTCTGGTATTATAGGTACAATATTTTCTATGATTATA
AGGTTAGAGTTAGCTGCTCCAGGCTCTCAAATACTGGGCGGTAATAGTCAACTTTATAAT
GTTATTATTACAGCTCACGCTTTTGTTATGATTTTCTTTTTTGTTATGCCTGTTATGATAGG
CGGTTTTGGGAATTGGTTTGTCCCTTTAATGATTGGTGCTCCTGATATGGCTTTTCCTCGA
CTAAATAATATAAGTTTTTGGTTATTGCCCCCTTCTTTATTCTTATTATTATGTTCTTCATT
AGTAGAGTTTGGTGCTGGTACTGGATGAACAGTTTATCCACCATTAAGTTCAATTGTAGC
TCATTCAGGCGGTTCGGTAGACTTAGCTATATTTAGTTTACATCTTGCTGGTATATCTTCT
CTTCTAGGTGCTATAAATTTTATTACTACAATATTTAATATGAGAGTGCCTGGTTTGTCAA
TGCATAAGTTACCGTTGTTTGTTTGATCTGTGCTAATTACAGCTTTTTTATTATTATTTTCT
TTACCTGTGTTAGCCGGCGCTATTACTATGCTTTTAACAGATAGAAATTTTAATACTAGTT
TTTTTGATCCATCTGGAGGTGGTGACCCTATTCTTTATCAAACATT

Cow1.2 COI (*Vannella*)

TTTTCGGTAATAATACGAATGGAGTTAGCTTTGCCTGGTAATCAAATTTTGCTAGTAATCA
TCAATTATATAATGTTATAGTTACTGCACATGCTGTTATTATGATTTTTTTTATGGTAATG
CCAGCGATGATAGGAGGTTTGGTAATATCTTTGTTCCTATTATGATAGGAGCTCCGGATA
TGGCTTTTCCTCGTCTTAATAATATTAGTTTTTGGCTTTTACCACCCTCATTTTTCTTGTTA
TTATATCTTCTTTTGTTGAGGGTGGTGCAGGGACTGGGTGGACTATTTATCCTCCTTTATC
AAGTATTGAAAGTCATTCAGGAGGGTCTGTAGATCTTGTCATATTTAGTTTACATTTAGC
AGGGTTTCATCTTTATTAGGTGCTATTAATTTCATAACAACAATATTAAATATGAGAGTA
AATCATATGACTTTTAGTCGTATTCCTCTTTTTGTTTGGGCAGTGTTTATAACTGCGTTTTT
ATTA

LN COI (*Vannella*)

ATAAAGGATCTCCCCCTCCTGTTGGATCAAAAAAAGTAGTATTAAAATTACGATCTGTTA
ATAACATAGTGTATAGCGCCGGCAAATACAGGTAATGATATTAATAATAAAAAAGCGGT
TATAAATACAGACCATACAAATAAAGGTAATCTTGACCAAAACATATTAGGTAATCTCAT
ATTTATATATGGTAGTTATAAAATTTAAAGCACCTAATATTGAAGAAATCCCTGATAAAT
GTAAACTAAAGATAGCTAAATCTACAGAAGGTCCTGAATGACCTACTATTGAAGATAAA
GGTGGATATACAGTCCAACCAGTACCAGCTCCGTTTTCAATTAAAGATGATGATAATAAT
AAAAAAAAAGATGGTGGTAATAACCAGAAGCTTATATTATTTAAACGAGGAAATGCCAT
ATCAGGCAGCTCCTATCATTAAAGGTACAAACCAATTACCAAATCCCCCTATTAAAGCTG
GCATTACCATAAAAAAAATCATAATAAAAGCATGTGCTGTTATTATAACATTATAAAATT
GATAATTATCAAATAAAATATTATCTCCTGGAGATGCTAATTCTATTCTAATTAAAACAG
ATAATAAAGTACCTATAATACCGGCAATACCCGC

Appendix 4. Differential expression analysis R Studio Script (modified from Edinburgh Genomics RNAseq Workshop)

```
#Open libraries(dplyr) (ggplot2) (rtracklayer) (readr) (BiocInstaller) (Rsubread)
(edgeR) (locfit) (statmod) (pheatmap)

rm(list =ls())
setwd('~/BAM/')
data_dir <- '53BAM'
geninfo <- '53Xdata2.tsv'

targets <- read.delim(file.path(geninfo), stringsAsFactors = FALSE)
targets$Id <- make.unique(targets$Name)
rownames(targets) <- targets$Id

bam_dir <- file.path(data_dir)
bam_files <- list.files(bam_dir, full.names = TRUE, pattern = '.bam')
bam_files

gtf_file <-
file.path('Acanthamoeba_castellanii_str_neff.Acastellanii.strNEFF_v1.37.gtf')

cat(readLines(gtf_file, n = 10), sep="\n")

gtf_content <- import(gtf_file, feature.type = 'gene')
gtf_content

annotation <- data.frame(elementMetadata(gtf_content), stringsAsFactors = FALSE)
rownames(annotation) <- annotation$gene_id

count_results <- featureCounts(files = bam_files, annot.ext = gtf_file,
isGTFAnnotationFile = TRUE, GTF.featureType = 'exon', GTF.attrType = 'gene_id',
strandSpecific = 2, isPairedEnd = FALSE, nthreads = 1)

colnames(count_results$counts) <- sub('.*(53.*)\\.bam', '\\1',
colnames(count_results$counts))
colnames(count_results$stat)[-1] <- sub('.*(53.*)\\.bam', '\\1',
colnames(count_results$stat)[-1])

raw_counts <- count_results$counts
head(raw_counts)
nrow(raw_counts)
length(which(rowSums(raw_counts>=1)>1))

count_results$stat

#This is not necessary but helps visualize
stats <- data.frame(count_results$stat[, -1], row.names = count_results$stat[, 1])
stats <- stats[apply(stats, 1, function(x) any(x > 0)),]
```

```
stats <- reshape2::melt(t(stats))
stats$name <- targets[stats$Var1, 'Name']

ggplot(stats, aes(x = Var1, y = value, fill = Var2)) +
  geom_bar(stat = "identity", position = 'fill') + theme_bw() + theme(axis.text.x =
element_text(angle = 90), legend.title = element_blank()) + xlab("Sample") +
ylab(NULL)

raw_counts <- raw_counts[rownames(annotation), rownames(targets)]

dgList <- DGEList(
  counts = raw_counts,
  group = targets$Hour,
  genes = annotation)
dgList

dgList$samples$Name <- targets$Name

#Normalisation

dgList <- calcNormFactors(dgList)

dgList$samples

#Explore the data

plotMDS(
  dgList,
  gene.selection ='pairwise',
  col = as.integer(dgList$samples$group),
  labels = dgList$samples$group
  )

#Filter the data

dgList <- dgList[rowSums(cpm(dgList)>=0.1) >= 10 , ,
      keep.lib.sizes=FALSE]
dgList <- calcNormFactors(dgList)

nrow(dgList)

#Explore the data

plotMDS(
  dgList,
  gene.selection ='pairwise',
  col = as.integer(dgList$samples$group),
  labels = dgList$samples$group
)

#Plotting a function
plot_gene <- function(gene_id, inputDgList){
  expr <- cpm(inputDgList)
```

```
  plot_data <- cbind(inputDgList$samples, expression = expr[gene_id,])
  plot_data <- plot_data[order(plot_data$group),]
  plot_data$sample <- factor(rownames(plot_data), levels = rownames(plot_data))
  p <- ggplot(plot_data, aes(x= Name, y= expression, fill= group)) + geom_bar(stat =
"identity") +
    theme_bw() +
    theme(axis.text.x = element_text(angle = 90, hjust = 1)) +
    ggtitle(paste(gene_id, annotation$gene_name[match(gene_id,
annotation$gene_id)], sep=": "))
  print(p)
}

plot_gene('ACA1_075240', dgList)

#Buidling a design matrix
design <- model.matrix(~ 0 + group, data = dgList$samples)
design

#Estimating dispersion
dgGlm <- estimateDisp(dgList, design, robust = TRUE)
plotBCV(dgGlm)

fit <- glmQLFit(dgGlm, design, robust = TRUE)
plotQLDisp(fit)

#Test for DE genes
contrasts <- data.frame(variable='group', group1='0', group2='24', group3='48',
group4='72', stringsAsFactors = FALSE)
contrasts

contrast_names <- apply(contrasts, 1, function(contrast)
paste(paste0(contrast[1], make.names(contrast[2])), paste0(contrast[1],
        make.names(contrast[3])), paste0(contrast[1],
        make.names(contrast[4])), paste0(contrast[1],
        make.names(contrast[5])), sep="-"))
contrast_names

contrast.matrix <- makeContrasts(contrasts = contrast_names, levels = design)
contrast.matrix

de <- glmQLFTest(fit, contrast = contrast.matrix[,1])

top_genes <- topTags(de, n=50)
top_genes

#Test different genes with plot_gene
plot_gene('ACA1_257600', dgList)

#Heatmap
plot_heatmap <- function(plot_genes, title= '', inputDgList){
  annotation_col <- inputDgList$samples[with(inputDgList$samples, order(group,
Name)), c('Name', 'group')]
  plot_genes <- plot_genes[plot_genes %in% rownames(inputDgList)]
```

```
  expression <- cpm(inputDgList)[plot_genes, rownames(annotation_col)]
  plotmatrix <- log2(expression + 0.1)
  rownames(plotmatrix) <- annotation[rownames(plotmatrix), 'gene_id']
  grid::grid.newpage()
  pheatmap(
    plotmatrix,
    show_rownames = T,
    annotation_col = annotation_col,
    border_color = NA,
    legend = FALSE,
    cluster_cols = FALSE,
    cluster_rows = TRUE,
    scale = 'row',
    color = colorRampPalette(rev(RColorBrewer::brewer.pal(n = 7, name =
"RdYlBu")))(100),
    main = title
  )
}

plot_heatmap(rownames(top_genes), inputDgList =  dgList)

#Extracting results
results <- topTags(de , n=nrow(dgList), sort.by = 'none')$table
results <- results[rownames(dgList),]

write.csv(results, file = 'Differential_results_53.csv')

#Filtering significant genes
fdr_treshold <- 0.05
fc_treshold <- 2

diffexp_genes <- rownames(results)[abs(results$logFC) >= log2(fc_treshold) &
results$FDR <= fdr_treshold ]

print(paste(length(diffexp_genes), 'genes are differentially expressed at fold change
of at least', fc_treshold, 'and a maximum FDR of', fdr_treshold))

#Visualising results
#Volcano plot
volcano_plot <- function(results_table, fc_treshold, fdr_treshold,
                         log=FALSE){
  results_table$significant <- 'no'
  results_table$significant[ abs(results_table$logFC) >=
                               log2(fc_treshold) & results_table$FDR <= fdr_treshold ] <- 'yes'
  if(! log){
    results_table$logFC <- sign(results_table$logFC)* 2^abs(results_table$logFC)
  }
  ggplot(results_table, aes(logFC, -log10(FDR), color=significant)) +
    geom_point(alpha = 0.5) +
    scale_colour_manual(name = 'significant', values = setNames(c('red',
'grey'),c('yes','no'))) +
    theme_bw()
  }
```

```
volcano_plot(results, fc_treshold, fdr_treshold)

volcano_plot(results, fc_treshold, fdr_treshold, log = TRUE)

#Analysis by gene sets using GAGE (Generally Applicable Gene-set Enrichment for
Pathway Analysis)
kg.acan=kegg.gsets("acan")
kegg.gs=kg.acan$kg.sets[kg.acan$sigmet.idx]
save(kegg.gs, file="kegg.hsa.sigmet.gsets.RData")

sets_name_to_id <- function(gene_sets){
  gene_sets_by_id <- lapply(gene_sets, function(x){
    gene_ids <- rownames(annotation)[match(geneIds(x), annotation$gene_name)]
    unique(gene_ids[! is.na(gene_ids)])
  })
  names(gene_sets_by_id) <- names(gene_sets)
  gene_sets_by_id
}

kegg.gs <- sets_name_to_id(kegg.gs)

setres_kegg <- mroast(
  y=dgGlm,
  index = ids2indices(kegg.gs, rownames(dgGlm)),
  design = design,
  contrast = contrast.matrix[,1],
  nrot=99999
)

setres_kegg <- setres_kegg[order(setres_kegg$FDR),]

setres_kegg_camera <- camera(
  y=dgGlm,
  index=ids2indices(kegg, rownames(dgGlm)),
  design=design,
  contrast=contrast.matrix[,1])
setres_kegg_camera <- setres_kegg_camera[order(setres_kegg_camera$FDR),]

barcode_plot <- function(gene_sets, gene_set, input_results){
  gene_ids <- rownames(input_results)
  fold_changes <- sign(input_results$logFC) * 2^abs(input_results$logFC)
  set_gene_ids <- gene_sets[[gene_set]]
  limma::barcodeplot(fold_changes, index = gene_ids %in% set_gene_ids, main =
gene_set)
}

barcode_plot(kegg.gs, rownames(setres_kegg)[1], results)

setres_kegg

write.csv(setres_kegg, file = 'pathways_all.csv')
```

Appendix 5. Top 50 DE genes for each pair comparison

Top 50 DE genes: 0 to 24 hours of encystment

Gene ID	LogFC	Product Description
ACA1_188370	10.0624	hypothetical protein
ACA1_365840	7.4465	hypothetical protein
ACA1_309360	6.4747	hypothetical protein
ACA1_265920	5.3974	hypothetical protein
ACA1_366590	5.1573	regulator of chromosome condensation (RCC1) repeat domain containing protein
ACA1_062050	5.0119	chondroitinase B
ACA1_238120	4.6957	RFX DNAbinding domain containing protein
ACA1_315750	4.6283	hypothetical protein
ACA1_158840	4.4655	metal dependent phosphohydrolase
ACA1_385740	4.2571	hypothetical protein
ACA1_065450	3.7461	sodium Ptype ATPase
ACA1_063060	3.0288	Ras family GTP-binding protein YPT1
ACA1_385350	-2.9259	HMG (high mobility group) box domain containing protein
ACA1_257540	-3.2669	ribonuclease, T2 family
ACA1_278160	-3.4047	hypothetical protein
ACA1_219500	-3.4093	alternative oxidase isoform B, putative
ACA1_026990	-3.4718	ABC2 type transporter superfamily protein
ACA1_278150	-3.6130	hypothetical protein
ACA1_069790	-3.6395	PPOD1 peroxidase
ACA1_194930	-3.7069	matrix attachment region binding protein
ACA1_066840	-3.7461	SNF2 domain containing protein
ACA1_115670	-4.0160	aconitate hydratase, mitochondrial
ACA1_130840	-4.0244	glycerol-3-phosphate dehydrogenase protein, putative
ACA1_153690	-4.1068	hypothetical protein
ACA1_127890	-4.1584	spermidine synthase
ACA1_269350	-4.1682	phosphoribosylformylglycinamidine synthase
ACA1_321760	-4.1894	haloacid dehalogenaselike hydrolase domain containing protein
ACA1_106370	-4.2367	glyoxylate reductase
ACA1_383580	-4.2995	sulfate adenylyltransferase
ACA1_048170	-4.3009	hydroxymethylglutarylCoA synthase
ACA1_186030	-4.4987	hypothetical protein
ACA1_093320	-4.5122	hypothetical protein
ACA1_362140	-4.5326	hypothetical protein
ACA1_356170	-4.5558	hypothetical protein
ACA1_295280	-4.5789	hypothetical protein
ACA1_002740	-4.7230	kinesin motor domain containing protein
ACA1_187750	-4.9225	chorismate mutase subfamily protein
ACA1_274380	-4.9538	protein kinase domain containing protein
ACA1_015330	-4.9690	CBS domain containing protein
ACA1_080050	-5.0751	DNAdirected RNA polymerase, omega subunit
ACA1_046460	-5.0850	ABC2 type transporter superfamily protein
ACA1_369120	-5.1743	proliferating cell nuclear antigen, Nterminal domain containing protein
ACA1_073360	-5.3755	RNA recognition motif domain containing protein
ACA1_227710	-5.3873	hypothetical protein
ACA1_214640	-5.4066	cobalaminindependent methionine synthase
ACA1_172200	-5.4433	hypothetical protein

ACA1_333720	-5.6512	hypothetical protein
ACA1_077280	-6.0560	CBS domain containing protein
ACA1_282980	-7.2319	hypothetical protein
ACA1_245710	-7.5805	hypothetical protein

Top 50 DE genes: 0 to 48 hours of encystment

Gene ID	LogFC	Product Description
ACA1_365350	6.2081	Ubiquitinconjugating enzyme subfamily protein
ACA1_309360	5.8617	hypothetical protein
ACA1_158840	5.8241	metal dependent phosphohydrolase
ACA1_062050	5.4005	chondroitinase B
ACA1_315750	4.4683	hypothetical protein
ACA1_146640	4.3133	hypothetical protein
ACA1_077510	4.2383	hypothetical protein
ACA1_385740	4.0497	hypothetical protein
ACA1_143060	4.0128	hypothetical protein
ACA1_054280	3.8058	hypothetical protein
ACA1_065450	3.3266	sodium Ptype ATPase
ACA1_092650	3.2577	GTPase domain containing protein
ACA1_233440	3.0014	hypothetical protein
ACA1_189440	2.6447	von Willebrand factor type A domain containing protein
ACA1_063150	-2.8541	transporter, major facilitator family protein
ACA1_278160	-2.9012	hypothetical protein
ACA1_325510	-2.9971	actin bundling protein
ACA1_278150	-3.0858	hypothetical protein
ACA1_154680	-3.1978	glutamate synthase, NADH/NADPH, small subunit
ACA1_048170	-3.3854	hydroxymethylglutarylCoA synthase
ACA1_194930	-3.4691	matrix attachment region binding protein
ACA1_172190	-3.5660	formin domain containing protein
ACA1_115670	-3.5737	aconitate hydratase, mitochondrial
ACA1_383580	-3.6756	sulfate adenylyltransferase
ACA1_144890	-3.7459	phosphoenolpyruvate phosphomutase
ACA1_106370	-3.7616	glyoxylate reductase
ACA1_130840	-3.8123	glycerol-3-phosphate dehydrogenase protein, putative
ACA1_144900	-3.8575	phosphoenolpyruvate phosphomutase
ACA1_385830	-3.9966	Microtubule associated protein (MAP65/ASE1 family)
ACA1_066840	-4.0982	SNF2 domain containing protein
ACA1_077280	-4.1058	CBS domain containing protein
ACA1_046460	-4.1635	ABC2 type transporter superfamily protein
ACA1_119180	-4.3269	ribonucleosidediphosphate reductase, alpha subunit
ACA1_002740	-4.4030	kinesin motor domain containing protein
ACA1_215650	-4.4166	hypothetical protein
ACA1_172200	-4.5118	hypothetical protein
ACA1_295280	-4.5231	hypothetical protein
ACA1_073360	-4.5962	RNA recognition motif domain containing protein
ACA1_362140	-4.8982	hypothetical protein
ACA1_080050	-4.9418	DNAdirected RNA polymerase, omega subunit
ACA1_186030	-5.0510	hypothetical protein
ACA1_132680	-5.4334	hypothetical protein
ACA1_071450	-5.5566	glycosyl transferase
ACA1_227710	-5.7236	hypothetical protein
ACA1_356170	-5.7677	hypothetical protein
ACA1_369120	-5.7745	proliferating cell nuclear antigen, Nterminal domain containing protein
ACA1_274380	-5.9591	protein kinase domain containing protein
ACA1_387270	-6.5685	hypothetical protein
ACA1_084830	-6.7496	hypothetical protein
ACA1_245710	-7.6091	hypothetical protein

Top 50 DE genes: 0 to 72 hours of encystment

Gene ID	LogFC	Product Description
ACA1_158980	5.7385	GATA zinc finger domain containing protein
ACA1_309360	5.5925	hypothetical protein
ACA1_184250	4.5000	SPFH domain / Band 7 domain containing protein
ACA1_158840	4.2536	metal dependent phosphohydrolase
ACA1_315750	4.0099	hypothetical protein
ACA1_202420	3.8941	hypothetical protein
ACA1_211500	3.7218	RAB5B protein, putative
ACA1_062050	3.6245	chondroitinase B
ACA1_065450	3.5433	sodium Ptype ATPase
ACA1_385740	3.4346	hypothetical protein
ACA1_159070	3.4326	mucin, putative
ACA1_183970	3.4119	hypothetical protein
ACA1_159080	3.3251	papain family cysteine protease subfamily protein
ACA1_206040	2.8698	MORN repeat-containing protein
ACA1_115650	2.7700	hypothetical protein
ACA1_186700	2.7251	ADPribosylation factor subfamily protein
ACA1_088960	2.5034	betalactamase
ACA1_092760	2.4726	hypothetical protein
ACA1_063060	2.3954	Ras family GTP-binding protein YPT1
ACA1_382920	2.3016	Ras subfamily protein
ACA1_263040	-2.0486	kinesin motor domain containing protein
ACA1_058800	-2.1763	mitochondrial 2oxodicarboxylate carrier 1, putative
ACA1_036530	-2.2721	transmembrane protein
ACA1_360600	-2.3029	hypothetical protein
ACA1_278160	-2.4700	hypothetical protein
ACA1_278150	-2.5113	hypothetical protein
ACA1_071650	-2.5596	acetoacetate decarboxylase
ACA1_076220	-2.8460	CBS domain containing protein
ACA1_097300	-2.8467	serine/threonine kinase
ACA1_154680	-2.9556	glutamate synthase, NADH/NADPH, small subunit
ACA1_175240	-3.0130	Rab32 protein, putative
ACA1_229500	-3.0614	homeobox domain containing protein
ACA1_400380	-3.4438	3deoxy-7-phosphoheptulonate synthase
ACA1_065860	-3.4575	ATPase, AAA domain containing protein
ACA1_069790	-3.5365	PPOD1 peroxidase
ACA1_215490	-3.7203	serine/threonine protein kinase 6, putative
ACA1_099250	-3.7736	hypothetical protein
ACA1_130840	-3.8171	glycerol-3-phosphate dehydrogenase protein, putative
ACA1_343310	-4.1617	NADH dehydrogenase, extrinsic, putative
ACA1_132680	-4.3708	hypothetical protein
ACA1_192710	-4.8644	ATPase/histidine kinase/DNA gyrase B/HSP90 domain containing protein
ACA1_285200	-4.8832	transporter, monovalent cation:proton antiporter2 (CPA2) family protein
ACA1_362140	-5.1430	hypothetical protein
ACA1_175880	-5.3797	hypothetical protein
ACA1_275730	-5.7902	phosphoglycerate mutase family domain containing protein
ACA1_289630	-6.2542	hypothetical protein
ACA1_377440	-6.3379	hypothetical protein
ACA1_207480	-6.6747	exported protein, putative
ACA1_084830	-7.0443	hypothetical protein
ACA1_245710	-7.6931	hypothetical protein

Top 50 DE genes: 24 to 48 hours of encystment

Gene ID	LogFC	Product Description
ACA1_365350	6.2081	Ubiquitinconjugating enzyme subfamily protein
ACA1_309360	5.8617	hypothetical protein
ACA1_158840	5.8241	metal dependent phosphohydrolase
ACA1_062050	5.4005	chondroitinase B
ACA1_315750	4.4683	hypothetical protein
ACA1_146640	4.3133	hypothetical protein
ACA1_077510	4.2383	hypothetical protein
ACA1_385740	4.0497	hypothetical protein
ACA1_143060	4.0128	hypothetical protein
ACA1_054280	3.8058	hypothetical protein
ACA1_065450	3.3266	sodium Ptype ATPase
ACA1_092650	3.2577	GTPase domain containing protein
ACA1_233440	3.0014	hypothetical protein
ACA1_189440	2.6447	von Willebrand factor type A domain containing protein
ACA1_063150	-2.8541	transporter, major facilitator family protein
ACA1_278160	-2.9012	hypothetical protein
ACA1_325510	-2.9971	actin bundling protein
ACA1_278150	-3.0858	hypothetical protein
ACA1_154680	-3.1978	glutamate synthase, NADH/NADPH, small subunit
ACA1_048170	-3.3854	hydroxymethylglutarylCoA synthase
ACA1_194930	-3.4691	matrix attachment region binding protein
ACA1_172190	-3.5660	formin domain containing protein
ACA1_115670	-3.5737	aconitate hydratase, mitochondrial
ACA1_383580	-3.6756	sulfate adenylyltransferase
ACA1_144890	-3.7459	phosphoenolpyruvate phosphomutase
ACA1_106370	-3.7616	glyoxylate reductase
ACA1_130840	-3.8123	glycerol-3-phosphate dehydrogenase protein, putative
ACA1_144900	-3.8575	phosphoenolpyruvate phosphomutase
ACA1_385830	-3.9966	Microtubule associated protein (MAP65/ASE1 family)
ACA1_066840	-4.0982	SNF2 domain containing protein
ACA1_077280	-4.1058	CBS domain containing protein
ACA1_046460	-4.1635	ABC2 type transporter superfamily protein
ACA1_119180	-4.3269	ribonucleosidediphosphate reductase, alpha subunit
ACA1_002740	-4.4030	kinesin motor domain containing protein+A3:C37A3:C42A1A3:CA3:C24
ACA1_215650	-4.4166	hypothetical protein
ACA1_172200	-4.5118	hypothetical protein
ACA1_295280	-4.5231	hypothetical protein
ACA1_073360	-4.5962	RNA recognition motif domain containing protein
ACA1_362140	-4.8982	hypothetical protein
ACA1_080050	-4.9418	DNAdirected RNA polymerase, omega subunit
ACA1_186030	-5.0510	hypothetical protein
ACA1_132680	-5.4334	hypothetical protein
ACA1_071450	-5.5566	glycosyl transferase
ACA1_227710	-5.7236	hypothetical protein
ACA1_356170	-5.7677	hypothetical protein
ACA1_369120	-5.7745	proliferating cell nuclear antigen, Nterminal domain containing protein
ACA1_274380	-5.9591	protein kinase domain containing protein
ACA1_387270	-6.5685	hypothetical protein
ACA1_084830	-6.7496	hypothetical protein
ACA1_245710	-7.6091	hypothetical protein

Top 50 DE genes: 24 to 72 hours of encystment

Gene ID	LogFC	Product Description
ACA1_158980	5.7385	GATA zinc finger domain containing protein
ACA1_309360	5.5925	hypothetical protein
ACA1_184250	4.5000	SPFH domain / Band 7 domain containing protein
ACA1_158840	4.2536	metal dependent phosphohydrolase
ACA1_315750	4.0099	hypothetical protein
ACA1_202420	3.8941	hypothetical protein
ACA1_211500	3.7218	RAB5B protein, putative
ACA1_062050	3.6245	chondroitinase B
ACA1_065450	3.5433	sodium Ptype ATPase
ACA1_385740	3.4346	hypothetical protein
ACA1_159070	3.4326	mucin, putative
ACA1_183970	3.4119	hypothetical protein
ACA1_159080	3.3251	papain family cysteine protease subfamily protein
ACA1_206040	2.8698	MORN repeat-containing protein
ACA1_115650	2.7700	hypothetical protein
ACA1_186700	2.7251	ADPribosylation factor subfamily protein
ACA1_088960	2.5034	betalactamase
ACA1_092760	2.4726	hypothetical protein
ACA1_063060	2.3954	Ras family GTP-binding protein YPT1
ACA1_382920	2.3016	Ras subfamily protein
ACA1_263040	-2.0486	kinesin motor domain containing protein
ACA1_058800	-2.1763	mitochondrial 2oxodicarboxylate carrier 1, putative
ACA1_036530	-2.2721	transmembrane protein
ACA1_360600	-2.3029	hypothetical protein
ACA1_278160	-2.4700	hypothetical protein
ACA1_278150	-2.5113	hypothetical protein
ACA1_071650	-2.5596	acetoacetate decarboxylase
ACA1_076220	-2.8460	CBS domain containing protein
ACA1_097300	-2.8467	serine/threonine kinase
ACA1_154680	-2.9556	glutamate synthase, NADH/NADPH, small subunit
ACA1_175240	-3.0130	Rab32 protein, putative
ACA1_229500	-3.0614	homeobox domain containing protein
ACA1_400380	-3.4438	3deoxy-7-phosphoheptulonate synthase
ACA1_065860	-3.4575	ATPase, AAA domain containing protein
ACA1_069790	-3.5365	PPOD1 peroxidase
ACA1_215490	-3.7203	serine/threonine protein kinase 6, putative
ACA1_099250	-3.7736	hypothetical protein
ACA1_130840	-3.8171	glycerol-3-phosphate dehydrogenase protein, putative
ACA1_343310	-4.1617	NADH dehydrogenase, extrinsic, putative
ACA1_132680	-4.3708	hypothetical protein
ACA1_192710	-4.8644	ATPase/histidine kinase/DNA gyrase B/HSP90 domain containing protein
ACA1_285200	-4.8832	transporter, monovalent cation:proton antiporter2 (CPA2) family protein
ACA1_362140	-5.1430	hypothetical protein
ACA1_175880	-5.3797	hypothetical protein
ACA1_275730	-5.7902	phosphoglycerate mutase family domain containing protein
ACA1_289630	-6.2542	hypothetical protein
ACA1_377440	-6.3379	hypothetical protein
ACA1_207480	-6.6747	exported protein, putative
ACA1_084830	-7.0443	hypothetical protein
ACA1_245710	-7.6931	hypothetical protein

Top 50 DE genes: 48 to 72 hours of encystment

Gene ID	LogFC	Product Description
ACA1_115650	1.4061	hypothetical protein
ACA1_125420	-1.0907	hypothetical protein
ACA1_364220	-1.2103	hypothetical protein
ACA1_391400	-1.2133	hypothetical protein
ACA1_064210	-1.3228	phospholipase D active site domain containing protein
ACA1_069480	-1.3413	transporter, major facilitator subfamily protein
ACA1_315630	-1.4364	oxidoreductase, zincbinding dehydrogenase superfamily protein
ACA1_064200	-1.4476	PH domain containing protein
ACA1_057230	-1.4784	Ubox domain containing protein
ACA1_097300	-1.5070	serine/threonine kinase
ACA1_290290	-1.5214	PB1 domain containing protein
ACA1_088730	-1.5297	hypothetical protein
ACA1_058310	-1.5998	hypothetical protein
ACA1_362440	-1.6217	hypothetical protein
ACA1_109730	-1.6586	replication factor rfc1 c terminal domain containing protein
ACA1_331480	-1.6796	membraneassociated ring finger (C3HC4) 2, putative
ACA1_267300	-1.7901	inositol polyphosphate phosphatase, putative
ACA1_377500	-1.7950	peptidase family c78 protein
ACA1_058040	-1.9587	Mimivirus encoded protein, putative
ACA1_389310	-2.0482	hypothetical protein
ACA1_171810	-2.0564	zinc finger, C3HC4 type (RING finger) domain containing protein
ACA1_351920	-2.0717	von Willebrand factor type A domain containing protein
ACA1_058110	-2.1023	Mimivirus encoded protein, putative
ACA1_163480	-2.1152	hypothetical protein
ACA1_171800	-2.1617	hypothetical protein
ACA1_057380	-2.2155	Mimivirus encoded protein, putative
ACA1_099250	-2.2349	hypothetical protein
ACA1_105910	-2.2416	RFX DNAbinding domain containing protein
ACA1_171270	-2.3026	hypothetical protein
ACA1_078640	-2.3578	Bbox zinc finger domain containing protein
ACA1_372060	-2.3604	oxidoreductase
ACA1_098680	-2.3842	transporter, major intrinsic protein (MIP) superfamily protein
ACA1_031590	-2.4225	hypothetical protein
ACA1_110450	-2.4817	glutamine synthetase
ACA1_057330	-2.5201	Hypothetical protein
ACA1_224050	-2.5362	hypothetical protein
ACA1_088220	-2.6784	PQQ enzyme repeat domain containing protein
ACA1_031580	-2.6812	hypothetical protein
ACA1_136090	-2.7057	hypothetical protein
ACA1_365350	-2.7188	Ubiquitinconjugating enzyme subfamily protein
ACA1_088450	-2.7739	hypothetical protein
ACA1_246840	-2.9084	hypothetical protein
ACA1_171260	-2.9541	hypothetical protein
ACA1_206740	-2.9671	hypothetical protein
ACA1_232440	-3.1502	transporter, major facilitator subfamily protein
ACA1_206510	-3.1927	hypothetical protein
ACA1_155580	-3.2257	chitin synthase
ACA1_365840	-3.5736	hypothetical protein
ACA1_384080	-3.6207	PLAC8 family protein
ACA1_027760	-3.6461	hypothetical protein

Appendix 6. DE pathways from 0 to 24 h comparison

Metabolic Pathway	NGenes	PropDown	PropUp	Direction	PValue	FDR
Synthesis and degradation of ketone bodies	6	0.000	0.833	Up	1.00E-05	5.67E-05
Sulfur metabolism	10	0.200	0.800	Up	1.00E-05	5.67E-05
Biosynthesis of amino acids	76	0.145	0.763	Up	1.00E-05	5.67E-05
Spliceosome	93	0.108	0.699	Up	1.00E-05	5.67E-05
Pyrimidine metabolism	63	0.238	0.635	Up	1.00E-05	5.67E-05
RNA transport	137	0.248	0.584	Up	1.00E-05	5.67E-05
Purine metabolism	94	0.245	0.564	Up	1.00E-05	5.67E-05
RNA polymerase	26	0.231	0.538	Up	1.00E-05	5.67E-05
Glutathione metabolism	34	0.176	0.471	Up	1.00E-05	5.67E-05
Lysine biosynthesis	10	0.000	1.000	Up	2.00E-05	9.00E-05
Ribosome biogenesis in eukaryotes	69	0.101	0.870	Up	2.00E-05	9.00E-05
Phenylalanine, tyrosine and tryptophan biosynthesis	18	0.056	0.722	Up	2.00E-05	9.00E-05
Cysteine and methionine metabolism	38	0.211	0.658	Up	2.00E-05	9.00E-05
Biosynthesis of secondary metabolites	293	0.208	0.645	Up	2.00E-05	9.00E-05
Porphyrin and chlorophyll metabolism	18	0.222	0.611	Up	2.00E-05	9.00E-05
RNA degradation	55	0.200	0.545	Up	2.00E-05	9.00E-05
Nitrogen metabolism	11	0.455	0.545	Up	2.00E-05	9.00E-05
Citrate cycle (TCA cycle)	30	0.033	0.933	Up	3.00E-05	0.0001109
DNA replication	36	0.139	0.833	Up	3.00E-05	0.0001109
acan01130 Biosynthesis of antibiotics	187	0.203	0.701	Up	3.00E-05	0.0001109
acan00900 Terpenoid backbone biosynthesis	21	0.190	0.619	Up	3.00E-05	0.0001109
acan00062 Fatty acid elongation	15	0.133	0.600	Up	3.00E-05	0.0001109
acan01100 Metabolic pathways	724	0.301	0.519	Up	3.00E-05	0.0001109
acan00450 Selenocompound metabolism	11	0.182	0.727	Up	4.00E-05	0.0001488
acan00440 Phosphonate and phosphinate metabolism	12	0.083	0.667	Up	5.00E-05	0.0001765
acan00190 Oxidative phosphorylation	52	0.192	0.654	Up	5.00E-05	0.0001765
acan00650 Butanoate metabolism	10	0.100	0.800	Up	7.00E-05	0.000221

acan00600 Sphingolipid metabolism	20	0.350	0.650	Up	7.00E-05	0.000221
acan00592 alpha-Linolenic acid metabolism	20	0.550	0.250	Down	7.00E-05	0.000221
acan00730 Thiamine metabolism	11	0.091	0.545	Up	7.00E-05	0.000221
acan03430 Mismatch repair	24	0.083	0.792	Up	8.00E-05	0.0002391
acan00970 Aminoacyl-tRNA biosynthesis	40	0.200	0.675	Up	8.00E-05	0.0002391
acan04144 Endocytosis	136	0.625	0.154	Down	9.00E-05	0.0002627
acan02010 ABC transporters	15	0.067	0.533	Up	0.00011	0.000315
acan00310 Lysine degradation	28	0.250	0.571	Up	0.00012	0.0003351
acan00670 One carbon pool by folate	10	0.200	0.800	Up	0.00013	0.0003542
acan01210 2-Oxocarboxylic acid metabolism	25	0.160	0.600	Up	0.00014	0.0003722
acan00030 Pentose phosphate pathway	16	0.125	0.750	Up	0.00017	0.0004429
acan01212 Fatty acid metabolism	40	0.325	0.475	Up	0.00018	0.0004577
acan01200 Carbon metabolism	87	0.161	0.701	Up	0.00019	0.0004602
acan00630 Glyoxylate and dicarboxylate metabolism	30	0.167	0.700	Up	0.00019	0.0004602
acan00563 Glycosylphosphatidylinositol(GPI)-anchor biosynthesis	25	0.680	0.000	Down	0.00022	0.0005221
acan03420 Nucleotide excision repair	37	0.243	0.514	Up	0.00023	0.0005337
acan03010 Ribosome	96	0.083	0.865	Up	0.00024	0.0005448
acan00770 Pantothenate and CoA biosynthesis	10	0.500	0.200	Down	0.00029	0.000646
acan00640 Propanoate metabolism	21	0.190	0.619	Up	3.00E-04	0.0006541
acan00785 Lipoic acid metabolism	2	0.000	1.000	Up	0.00045	0.0009657
acan01040 Biosynthesis of unsaturated fatty acids	24	0.375	0.500	Up	0.00056	0.0011794
acan03410 Base excision repair	43	0.186	0.488	Up	6.00E-04	0.0012386
acan00790 Folate biosynthesis	25	0.280	0.560	Up	0.00063	0.001275
acan00130 Ubiquinone and other terpenoid-quinone biosynthesis	3	0.000	1.000	Up	0.00065	0.00129
acan00330 Arginine and proline metabolism	25	0.280	0.440	Up	0.00092	0.0017948
acan00562 Inositol phosphate metabolism	59	0.525	0.169	Down	0.00103	0.001938
acan00250 Alanine, aspartate and glutamate metabolism	34	0.324	0.500	Up	0.00104	0.001938
acan00620 Pyruvate metabolism	34	0.176	0.676	Up	0.00105	0.001938
acan03015 mRNA surveillance pathway	55	0.182	0.545	Up	0.00127	0.0022422
acan00071 Fatty acid degradation	30	0.500	0.267	Down	0.00127	0.0022422

acan00350 Tyrosine metabolism	16	0.125	0.563	Up	0.00128	0.0022422
acan00531 Glycosaminoglycan degradation	10	0.600	0.100	Down	0.00132	0.0022734
acan00511 Other glycan degradation	8	0.750	0.125	Down	0.00141	0.0023885
acan00460 Cyanoamino acid metabolism	11	0.091	0.727	Up	0.00149	0.0024831
acan00603 Glycosphingolipid biosynthesis - globo series	7	0.571	0.429	Down	0.00186	0.0030518
acan00100 Steroid biosynthesis	29	0.310	0.552	Up	0.00222	0.0035314
acan00260 Glycine, serine and threonine metabolism	29	0.345	0.655	Up	0.00225	0.0035314
acan00360 Phenylalanine metabolism	14	0.143	0.571	Up	0.00226	0.0035314
acan00430 Taurine and hypotaurine metabolism	8	0.375	0.375	Down	0.00229	0.0035314
acan00564 Glycerophospholipid metabolism	72	0.278	0.417	Up	0.0024	0.0036461
acan00010 Glycolysis / Gluconeogenesis	35	0.314	0.600	Up	0.00265	0.0039675
acan04070 Phosphatidylinositol signaling system	79	0.430	0.228	Down	0.00302	0.004457
acan00740 Riboflavin metabolism	5	0.400	0.400	Up	0.00309	0.0044953
acan03450 Non-homologous end-joining	8	0.375	0.375	Up	0.00325	0.0046618
acan00500 Starch and sucrose metabolism	29	0.310	0.586	Up	0.00344	0.0048663
acan00410 beta-Alanine metabolism	21	0.571	0.333	Down	0.00419	0.0058475
acan00380 Tryptophan metabolism	26	0.308	0.538	Up	0.00484	0.0066645
acan00750 Vitamin B6 metabolism	5	0.600	0.200	Down	0.00621	0.0084388
acan04120 Ubiquitin mediated proteolysis	97	0.505	0.216	Down	0.00729	0.0097772
acan00040 Pentose and glucuronate interconversions	13	0.615	0.385	Down	0.00898	0.011889
acan00232 Caffeine metabolism	5	0.200	0.400	Up	0.0108	0.0141165
acan04146 Peroxisome	79	0.418	0.367	Down	0.01111	0.0143381
acan04145 Phagosome	91	0.484	0.308	Down	0.01485	0.0187315
acan00561 Glycerolipid metabolism	47	0.340	0.298	Down	0.01488	0.0187315
acan00290 Valine, leucine and isoleucine biosynthesis	3	0.667	0.333	Down	0.01648	0.0204933
acan00340 Histidine metabolism	19	0.526	0.316	Down	0.01881	0.0231098
acan00510 N-Glycan biosynthesis	28	0.464	0.250	Down	0.02039	0.024558
acan03440 Homologous recombination	31	0.194	0.516	Up	0.02047	0.024558
acan00280 Valine, leucine and isoleucine degradation	34	0.412	0.412	Up	0.02404	0.0282962
acan00471 D-Glutamine and D-glutamate metabolism	3	0.333	0.333	Down	0.02414	0.0282962

acan00780 Biotin metabolism	3	0.667	0.333	Down	0.03294	0.0381747
acan03060 Protein export	18	0.222	0.500	Up	0.04633	0.0530916
acan03050 Proteasome	33	0.030	0.818	Up	0.04734	0.0536463
acan00760 Nicotinate and nicotinamide metabolism	15	0.467	0.400	Down	0.05198	0.0582577
acan00061 Fatty acid biosynthesis	10	0.400	0.500	Up	0.05354	0.059354
acan00051 Fructose and mannose metabolism	19	0.526	0.368	Down	0.05762	0.0631906
acan04141 Protein processing in endoplasmic reticulum	91	0.418	0.396	Up	0.09095	0.098685
acan03022 Basal transcription factors	24	0.208	0.208	Up	0.10552	0.1132898
acan00052 Galactose metabolism	18	0.389	0.556	Up	0.11862	0.1260284
acan00565 Ether lipid metabolism	32	0.313	0.375	Up	0.12772	0.1342982
acan04130 SNARE interactions in vesicular transport	30	0.367	0.233	Down	0.1754	0.182554
acan00590 Arachidonic acid metabolism	18	0.389	0.333	Down	0.23516	0.2422809
acan00520 Amino sugar and nucleotide sugar metabolism	34	0.412	0.412	Down	0.40469	0.4127787
acan04122 Sulfur relay system	7	0.429	0.429	Down	0.5954	0.60129
acan00053 Ascorbate and aldarate metabolism	13	0.462	0.385	Up	0.70729	0.70729

Appendix 7. List of enzymes reported from AmoebaDB and the DE results during the first 24 hours of encystment

The results are records found when searching for the following words: cellulases, lipases, amylases, proteases and lysozymes. The table includes the gene_ID and product description from AmoebaDB. They also include the LogFC and FDR for the comparison between 0 and 24 hours. The cells with a LogFC above 2 are marked in red.

Cellulases		**0vs24 hours**	
Gene ID	**Product Description**	**LogFC**	**FDR**
ACA1_251790	cellulase (glycosyl hydrolase family 5) subfamily protein	-0.010	0.953
ACA1_384790	cellulase (glycosyl hydrolase family 5) subfamily protein	0.708	0.001
ACA1_206090	cellulase	1.389	0.000
ACA1_206200	Cellulase	1.389	0.000
ACA1_370220	cellulase (glycosyl hydrolase family 5) subfamily protein	-2.662	0.000
ACA1_206340	cellulase (glycosyl hydrolase family 5) subfamily protein	1.985	0.007
ACA1_063240	cellulase (glycosyl hydrolase family 5) subfamily protein	-0.531	0.009
ACA1_133010	cellulase (glycosyl hydrolase family 5) subfamily protein	0.790	0.006
ACA1_087300	cellulase, putative	-0.042	0.924
ACA1_163320	cellulase (glycosyl hydrolase family 5) subfamily protein	0.507	0.034
ACA1_309350	cellulase (glycosyl hydrolase family 5) subfamily protein	-0.003	0.987
ACA1_364610	cellulase (glycosyl hydrolase family 5) subfamily protein	0.157	0.313
ACA1_244360	cellulase (glycosyl hydrolase family 5) subfamily protein	-3.015	0.012
ACA1_149090	betamannosidase	1.975	0.000
ACA1_364400	hypothetical protein	-1.143	0.000
ACA1_033490	glycoside hydrolase family protein	-1.684	0.000
ACA1_140950	multifunctional cellulase	-2.880	0.001
Amylases			
Gene ID	**Product Description**	**LogFC**	**FDR**
ACA1_074160	alpha amylase	-0.964	0.000
ACA1_295390	acidstable alpha-amylase	0.354	0.049
ACA1_165530	alpha amylase, catalytic subfamily protein	1.036	0.000
ACA1_391360	alpha amylase, putative	0.555	0.002
ACA1_400390	alpha amylase, catalytic subfamily protein	-0.060	0.763
ACA1_013390	glucan (1,4alpha-), branching enzyme 1, putative	-2.457	0.000
ACA1_254850	trehalose synthase	-0.289	0.053
Lipases			
Gene ID	**Product Description**	**LogFC**	**FDR**
ACA1_326860	lipase	-0.935	0.000
ACA1_291890	lipase	1.817	0.000
ACA1_295850	lipase	0.445	0.064
ACA1_175630	lipase	1.280	0.010
ACA1_069860	lipase	0.075	0.703
ACA1_384090	GDSLlike lipase/acylhydrolase domain containing protein	-0.188	0.376
ACA1_226740	GDSL family lipase/acylhydrolase domain containing protein	-0.281	0.122
ACA1_277840	lipase	-2.706	0.000
ACA1_339020	class 3 lipase	1.646	0.000
ACA1_116640	Lipase	-0.498	0.002
ACA1_201460	lipase	-0.095	0.646
ACA1_208460	abhydrolase associated lipase	-0.200	0.266

ACA1_059830	lipase	0.149	0.440
ACA1_060160	lipase	-1.281	0.000
ACA1_062000	lipase	-1.677	0.018
ACA1_062220	lipase, putative	-2.440	0.012
ACA1_062420	lipase	-1.328	0.103
ACA1_366510	lipase	0.555	0.001
ACA1_366890	lipase	0.499	0.111
ACA1_372580	lipase A precursor family protein	1.289	0.000
ACA1_256800	ab-hydrolase associated lipase region protein	2.176	0.000
ACA1_261060	lipase, putative	0.254	0.641
ACA1_145710	lipase	-0.876	0.000
ACA1_294900	cytohesin 2, putative	0.441	0.005
ACA1_019190	lipase/esterase, putative	0.366	0.010
ACA1_143690	transmembrane protein	2.943	0.000
ACA1_068240	endo-1,4-beta-glucanase, putative	-0.344	0.077
ACA1_357610	RasGEF domain containing protein	0.667	0.008
ACA1_253120	acyloxyacyl hydrolase	-1.144	0.000
ACA1_254720	GDSL-lilke lipase/acylhydrolase family protein	-0.283	0.270
ACA1_158780	hypothetical protein	-0.016	0.928
ACA1_384400	GDSLlike lipase/acylhydrolase domain containing protein	1.872	0.001
ACA1_277520	triacylglycerol lipase	0.189	0.281
ACA1_279590	hypothetical protein	0.178	0.491
ACA1_280270	Phospholipase	0.780	0.001
ACA1_113860	acyloxyacyl hydrolase	-0.489	0.000
ACA1_057010	dihydrofolate reductase	4.208	0.000
ACA1_028690	hydrolase, alpha/beta fold domain containing protein	1.834	0.000
ACA1_031640	hydrolase, alpha/beta fold domain containing protein	-0.239	0.104
ACA1_078900	unspecified product	0.441	0.016
ACA1_377540	triacylglycerol lipase	0.639	0.000
ACA1_212240	esterase/lipase (), putative	0.247	0.090
ACA1_380910	phospholipase a2, putative	-0.282	0.055
ACA1_374470	esterase/lipase/thioesterase family protein	-0.175	0.535
ACA1_256920	triacylglycerol lipase	2.842	0.000
ACA1_387610	unspecified product	0.785	0.000
Lysozyme			
Gene ID	**Product Description**	**LogFC**	**FDR**
ACA1_062600	Ctype lysozyme/alpha-lactalbumin superfamily protein	0.066	0.685
ACA1_069730	Lysozyme, putative	-0.673	0.000
ACA1_187480	hypothetical protein	-1.094	0.001
ACA1_215950	hypothetical protein	1.162	0.000
Proteases			
Gene ID	**Product Description**	**LogFC**	**FDR**
ACA1_018960	papain family cysteine protease subfamily protein	-0.698	0.000
ACA1_291590	Kazaltype serine protease inhibitor domain containing protein	1.386	0.032
ACA1_291610	Kazaltype serine protease inhibitor domain containing protein	3.542	0.000
ACA1_294930	Ulp1 protease family, Cterminal catalytic domain containing protein	-0.524	0.002
ACA1_295790	ubiquitin specific protease, putative	-0.872	0.000
ACA1_110420	CAAX amino terminal protease family protein	0.581	0.000
ACA1_173890	ubiquitinspecific protease	-0.929	0.000
ACA1_173980	OTU family cysteine protease	0.416	0.005
ACA1_176020	CAAX amino terminal protease family protein	0.801	0.000
ACA1_056670	cathepsin Llike cysteine protease	4.208	0.000
ACA1_085940	protease	-0.513	0.008
ACA1_153630	OTUlike cysteine protease family protein	0.643	0.001
ACA1_074920	OTUlike cysteine protease	1.419	0.000
ACA1_082960	ubiquitin specific protease 22 family protein	-1.073	0.082
ACA1_069520	ubiquitin specific protease 54 family protein	0.943	0.000

ACA1_253090	Ulp1 protease family, Cterminal catalytic domain containing protein	0.160	0.460
ACA1_255370	CAAX prenyl protease 2, putative	-1.044	0.001
ACA1_255720	eukaryotic aspartyl protease superfamily protein	-0.704	0.000
ACA1_159080	papain family cysteine protease subfamily protein	3.451	0.000
ACA1_022680	papain family cysteine protease subfamily protein	1.017	0.001
ACA1_046850	OTU family cysteine protease	0.502	0.303
ACA1_079690	papain family cysteine protease subfamily protein	0.418	0.556
ACA1_183400	ATPdependent protease HslVU, peptidase subunit	-0.484	0.001
ACA1_277940	cysteine protease 5, putative	0.925	0.000
ACA1_098800	ubiquitin specific protease 44 isoform 2, putative	-0.166	0.264
ACA1_184700	calpain family cysteine protease domain containing protein	0.308	0.035
ACA1_198270	papain family cysteine protease subfamily protein	-5.029	0.001
ACA1_198340	papain family cysteine protease subfamily protein	-0.454	0.002
ACA1_267130	OTU family cysteine protease	0.844	0.000
ACA1_270350	papain family cysteine protease containing protein	0.482	0.002
ACA1_236100	OTUlike cysteine protease protein	0.910	0.000
ACA1_236310	calpain family cysteine protease domain containing protein	-0.369	0.113
ACA1_151240	OTU family cysteine protease	-0.205	0.160
ACA1_138380	cathepsin Llike cysteine protease	6.260	0.000
ACA1_056940	cysteine protease	4.208	0.000
ACA1_060040	papain family cysteine protease	-0.249	0.635
ACA1_062700	Ulp1 protease family, Cterminal catalytic domain containing protein	0.126	0.544
ACA1_066290	papain family cysteine protease subfamily protein	-0.336	0.290
ACA1_304310	cysteine protease	-0.601	0.015
ACA1_383490	ATPdependent protease La, putative	-0.291	0.041
ACA1_192370	OTUlike cysteine protease domain containing protein	-0.364	0.017
ACA1_322170	papain family cysteine protease subfamily protein	-1.617	0.124
ACA1_244110	cysteine protease	-0.559	0.041
ACA1_301120	OTUlike cysteine protease	0.239	0.097
ACA1_190280	ubiquitin-specific protease, putative	0.226	0.336
ACA1_145750	Kazaltype serine protease inhibitor domain containing protein	-0.264	0.322
ACA1_288610	serpin (serine proteinase inhibitor) superfamily protein	1.043	0.000
ACA1_290220	ubiquitin carboxyl-terminal hydrolase	1.053	0.000
ACA1_290560	carboxypeptidase A3, putative	-0.490	0.001
ACA1_291510	Ubiquitin carboxylterminal hydrolase 15, putative	0.228	0.133
ACA1_291710	zinc metalloproteinase STE24, putative	-0.094	0.638
ACA1_293860	ubiquitin specific peptidase 16, putative	0.170	0.268
ACA1_296310	cysteine proteinase precursor, putative	-0.025	0.876
ACA1_296830	peptidase C19 family protein	-0.541	0.002
ACA1_110330	ubiquitin carboxyl-terminal hydrolase	0.856	0.000
ACA1_321400	encystationmediating serine proteinase	7.185	0.000
ACA1_170770	ubiquitin specific protease 39 and snrnp assembly factor, putative	-0.013	0.940
ACA1_173660	aspartic proteinase	-1.095	0.000
ACA1_322370	cathepsin L 2 precursor, putative	0.583	0.000
ACA1_169550	ubiquitin carboxyl-terminal hydrolase	0.946	0.026
ACA1_169560	ubiquitin interaction motif domain containing protein	0.753	0.011
ACA1_169590	ubiquitin interaction motif domain containing protein	0.648	0.025
ACA1_272730	Ubiquitin carboxylterminal hydrolase	-1.056	0.000
ACA1_074760	signalosome complex protein	0.246	0.094
ACA1_068540	Prokumamolisin, activation domain containing protein	-2.119	0.000
ACA1_072170	poly(A)specific ribonuclease subunit protein, putative	0.060	0.727
ACA1_072350	ubiquitin carboxyl-terminal hydrolase	0.562	0.000
ACA1_072480	hypothetical protein	0.213	0.345
ACA1_073080	26S proteasome regulatory complex subunit RPN11, putative	-0.569	0.001
ACA1_142300	eukaryotic translation initiation factor 3, subunit 3 gamma, 40kDa isoform 1, putative	-0.674	0.000
ACA1_251780	serpin (serine proteinase inhibitor) superfamily protein	-1.462	0.010

ACA1_157220	Prokumamolisin, activation domain containing protein	-1.301	0.000
ACA1_157540	Prokumamolisin, activation domain containing protein	-0.641	0.000
ACA1_022730	cathepsin Blike cysteine proteinase	0.462	0.190
ACA1_337290	serpin (serine proteinase inhibitor) superfamily protein	-0.153	0.767
ACA1_181520	cathepsin L, putative	0.079	0.760
ACA1_184160	26S protease regulatory subunit 6B family protein	-0.354	0.026
ACA1_184170	pathogenesisrelated protein PR-6 type, putative	-1.971	0.028
ACA1_184420	cathepsin Llike proteinase	-0.695	0.000
ACA1_277480	DegPtype protease	-0.304	0.038
ACA1_277790	protease, serine, 16 (thymus), putative	-0.247	0.094
ACA1_278460	alkaline serine protease	-1.005	0.000
ACA1_280300	protein kinase domain containing protein	0.022	0.910
ACA1_280510	hypothetical protein	0.164	0.304
ACA1_280570	Znfinger in Ran binding protein and others domain containing protein	0.491	0.001
ACA1_054220	MHCK/EF2 kinase domain containing protein	-2.261	0.000
ACA1_054320	PremRNA processing splicing factor 8, putative	-0.422	0.008
ACA1_317670	cathepsin L, putative	0.497	0.122
ACA1_030090	unspecified product	-1.710	0.000
ACA1_113780	COP9 signalosome subunit 6, putative	-0.022	0.893
ACA1_115390	cysteine proteinase	3.915	0.000
ACA1_197600	BRCA1/BRCA2containing complex subunit 3, putative	0.163	0.374
ACA1_198450	hypothetical protein	0.916	0.000
ACA1_200110	Prokumamolisin, activation domain containing protein	-1.244	0.000
ACA1_264710	membranebound transcription factor protease, site 1 isoform 1, putative	0.268	0.061
ACA1_266750	ubiquitin domain containing protein	-0.001	0.994
ACA1_269700	peptidase C56, PfpI, putative	-0.082	0.604
ACA1_336430	cysteine protease, putative	0.948	0.000
ACA1_232670	BAH domain containing protein	-0.004	0.980
ACA1_234230	peptidase, S8/S53 subfamily protein	-0.137	0.738
ACA1_234340	Ubiquitin carboxylterminal hydrolase domain containing protein	0.736	0.000
ACA1_040710	ATP-dependent metallopeptidase HflB subfamily protein	-1.056	0.000
ACA1_154200	Prokumamolisin, activation domain containing protein	-0.679	0.000
ACA1_155530	Cathepsin L precursor (Cysteine proteinase 1), putative	-0.842	0.011
ACA1_045340	26S proteasome regulatory subunit	-1.432	0.000
ACA1_314970	ubiquitin carboxylterminal hydrolase 9, putative	0.522	0.002
ACA1_119850	unspecified product	-0.574	0.012
ACA1_371700	Ubiquitin carboxylterminal hydrolase	-0.110	0.483
ACA1_372220	deubiquinating enzyme, putative	0.651	0.081
ACA1_208480	hypothetical protein	1.146	0.000
ACA1_057260	unspecified product	-2.628	0.000
ACA1_057280	peptidase, S8/S53 subfamily protein	0.519	0.033
ACA1_057490	zinc carboxypeptidase superfamily protein	-0.123	0.434
ACA1_061150	UBA/TSN domain containing protein	0.651	0.000
ACA1_061760	Serine protease precursor, putative	-0.930	0.000
ACA1_065810	serpin (serine proteinase inhibitor) superfamily protein	-1.084	0.006
ACA1_128830	encystationmediating serine proteinase	3.136	0.000
ACA1_382620	serpin (serine proteinase inhibitor) superfamily protein	-0.389	0.012
ACA1_087440	cysteine proteinase	-2.183	0.000
ACA1_087710	ICElike protease (caspase) p20 domain containing protein	0.550	0.186
ACA1_087870	Tripeptidylpeptidase 1, putative	-1.118	0.000
ACA1_348950	ubiquitin domain containing protein	0.361	0.011
ACA1_203510	ubiquitin carboxylterminal hydrolase 22, putative	-0.108	0.748
ACA1_178540	Ubiquitin carboxylterminal hydrolase	0.067	0.697
ACA1_222700	peptidase S8 and S53 subtilisin kexin sedolisin, putative	0.993	0.000
ACA1_223330	peptidase S8 and S53 subtilisin kexin sedolisin, putative	0.013	0.941
ACA1_321740	Prokumamolisin, activation domain containing protein	-0.670	0.000

ACA1_164210	hypothetical protein	0.335	0.020
ACA1_164780	ubiquitin domain containing protein	0.668	0.000
ACA1_164830	unspecified product	4.460	0.000
ACA1_341090	26S protease regulatory subunit 6a, putative	-0.285	0.062
ACA1_276790	zinc finger, C3HC4 type (RING finger) domain containing protein	-0.714	0.000
ACA1_216430	ubiquitin specific peptidase 38, putative	0.684	0.000
ACA1_220180	hypothetical protein	-0.295	0.037
ACA1_051490	Cysteine proteinase 5, putative	3.463	0.000
ACA1_391460	Ubiquitin carboxylterminal hydrolase	0.168	0.268
ACA1_125110	cysteine proteinase	-6.755	0.001
ACA1_077970	Prokumamolisin, activation domain containing protein	0.011	0.951
ACA1_107270	ATPdependent metallopeptidase HflB subfamily protein	-0.474	0.001
ACA1_365390	ubiquitin domain containing protein	0.567	0.000
ACA1_037710	unspecified product	-0.213	0.143
ACA1_038220	ubiquitin carboxyl-terminal hydrolase	0.586	0.001
ACA1_024230	subtilisin, putative	0.732	0.000
ACA1_020120	Prokumamolisin, activation domain containing protein	0.195	0.301
ACA1_244100	papain family cysteine protease subfamily protein	-1.600	0.008
ACA1_244230	cathepsin L, putative	-0.401	0.005
ACA1_100430	IMP2 inner mitochondrial membrane protease family isoform 2, putative	-0.494	0.029
ACA1_096350	cystatin, putative	-0.477	0.002
ACA1_096600	CUE domain containing protein	-1.102	0.000
ACA1_098100	serpin	-1.270	0.179
ACA1_098270	Prokumamolisin, activation domain containing protein	0.821	0.004
ACA1_329660	putative ubiquitin carboxylterminal hydrolase 11, putative	0.238	0.102
ACA1_101480	Prokumamolisin, activation domain containing protein	-0.434	0.023
ACA1_101540	ubiquitin carboxyl-terminal hydrolase	0.788	0.000
ACA1_399750	Carboxypeptidase A1, putative	0.263	0.066
ACA1_273880	encystationmediating serine proteinase	-0.645	0.001
ACA1_257970	Endoprotease FURIN, putative	0.273	0.231
ACA1_136150	26S proteasome nonATPase regulatory subunit 7, putative	-0.535	0.001
ACA1_387910	peptidase, S8/S53 subfamily protein	-0.454	0.001
ACA1_317520	cysteine protease atg4a, putative	-0.677	0.049
ACA1_337990	intracellular protease, PfpI family protein	0.657	0.000
ACA1_109090	Prokumamolisin, putative	-1.722	0.000

Bibliography

Abd, H., Saeed, A., Weintraub, A., Nair, G. B., & Sandström, G. (2007). *Vibrio cholerae* O1 strains are facultative intracellular bacteria, able to survive and multiply symbiotically inside the aquatic free-living amoeba *Acanthamoeba castellanii*. *FEMS Microbiology Ecology*, *60*(1), 33–39. https://doi.org/10.1111/j.1574-6941.2006.00254.x

Abergel, C., & Claverie, J. M. (2014). *Pithovirus sibericum*: réveil d'un virus géant de plus de 30 000 ans. *Medecine/Sciences*, *30*(3), 329–331. https://doi.org/10.1051/medsci/20143003022

Adam, K. M. G., Blewett, D. A., & Flamm, W. G. (1969). The DNA of *Acanthamoeba spp.*; a method for extraction and its characterization. *Journal of Protozoology*, *16*(1), 6–12.

Adl, S. M., Simpson, A. G. B., Farmer, M. A., Andersen, R. A., Anderson, O. R., Barta, J. R., … Taylor, M. F. J. R. (2005). The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. *Journal of Eukaryotic Microbiology*, *52*(5), 399–451. https://doi.org/10.1111/j.1550-7408.2005.00053.x

Aichelburg, A. C., Walochnik, J., Assadian, O., Prosch, H., Steuer, A., Perneczky, G., … Vetter, N. (2008). Successful treatment of disseminated *Acanthamoeba sp.* infection with miltefosine. *Emerging Infectious Diseases*, *14*(11), 1743–1746. https://doi.org/10.3201/eid1411.070854

Aitken, D., Hay, J., Kinnear, F. B., Kirkness, C. M., Lee, W. R., & Seal, D. V. (1996). Amebic keratitis in a wearer of disposable contact lenses due to a mixed *Vahlkampfia* and *Hartmannella infection*. *Ophthalmology*, *103*(3), 485–494. https://doi.org/10.1016/S0161-6420(96)30667-2

Aksozek, A., McClellan, K., Howard, K., Niederkorn, J. Y., & Alizadeh, H. (2002). Resistance of *Acanthamoeba castellanii* cysts to physical, chemical, and radiological conditions. *The Journal of Parasitology*, *88*(3), 621–623. https://doi.org/10.1645/0022-3395(2002)088[0621:ROACCT]2.0.CO;2

Alexandrakis, G., Miller, D., AW, H., Lin, J., Rapuano, C., Laibason, P., … Cohen, E. (1998). Amebic keratitis due to *Vahlkampfia* infection following corneal trauma. *Archives of Ophthalmology*, *116*(7), 950–951. Retrieved from http://dx.doi.org/

Ali, M. F., Lips, K. R., Knoop, F. C., Fritzsch, B., Miller, C., & Conlon, J. M. (2002). Antimicrobial peptides and protease inhibitors in the skin secretions of the crawfish frog, *Rana areolata*. *Biochimica et Biophysica Acta*, *1601*(1), 55–63.

Alizadeh, H., Apte, S., El-Agha, M.-S. H., Li, L., Hurt, M., Howard, K., … Niederkorn, J. Y. (2001). Tear IgA and serum IgG antibodies against *Acanthamoeba* in patients with *Acanthamoeba* keratitis. *Cornea*, *20*(6), 622–627. Retrieved from http://journals.lww.com/corneajrnl/Fulltext/2001/08000/Tear_IgA_and_Serum_IgG_Antibodies_Against.13.aspx

Alizadeh, H., Neelam, S., & Niederkorn, J. Y. (2007). Effect of immunization with the mannose-induced *Acanthamoeba* protein and *Acanthamoeba* plasminogen activator in mitigating *Acanthamoeba* keratitis. *Investigative Ophthalmology and Visual Science*, *48*(12), 5597–5604. https://doi.org/10.1167/iovs.07-0407

Alizadeh, H., Pidherney, M. S., McCulley, J. P., & Niederkorn, J. Y. (1994). Apoptosis as a mechanism of cytolysis of tumor cells by a pathogenic free- living amoeba. *Infection and Immunity*, *62*(4), 1298–1303.

Allen, P. G., & Dawidowicz, E. A. (1990). Phagocytosis in *Acanthamoeba*: I. A mannose receptor is responsible for the binding and phagocytosis of yeast. *Journal of Cellular Physiology*, *145*(3), 508–513. https://doi.org/10.1002/jcp.1041450317

Allombert, J., Vianney, A., Laugier, C., Petry, S., & Hébert, L. (2014). Survival of taylorellae in the environmental amoeba *Acanthamoeba castellanii*. *BMC Microbiology*, *14*(69). https://doi.org/10.1186/1471-2180-14-69

Alsam, S., Jeong, S. R., Sissons, J., Dudley, R., Kim, K. S., & Khan, N. A. (2006). Escherichia coli interactions with Acanthamoeba: a symbiosis with environmental and clinical implications. *Journal of Medical Microbiology*, *55*(Pt 6), 689–694. https://doi.org/10.1099/jmm.0.46497-0

Alsam, S., Sissons, J., Dudley, R., & Khan, N. A. (2005a). Mechanisms associated with *Acanthamoeba castellanii* (T4) phagocytosis. *Parasitology Research*, *96*(6), 402–409. https://doi.org/10.1007/s00436-005-1401-z

Alsam, S., Sissons, J., Jayasekera, S., & Khan, N. A. (2005b). Extracellular proteases of Acanthamoeba castellanii (encephalitis isolate belonging to T1 genotype) contribute to increased permeability in an in vitro model of the human blood-brain barrier. *Journal of Infection*, *51*, 150–156. https://doi.org/10.1016/j.jinf.2004.09.001

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Amann, R., Springer, N., Schönhuber, W., Ludwig, W., Schmid, E. N., Müller, K. D., & Michel, R. (1997). Obligate intracellular bacterial parasites of a Acanthamoebae related to *Chlamydia spp. Applied and Environmental Microbiology*, *63*(1), 115–121.

Amaral-Zettler, L. A., Cole, J., Laatsch, A. D., Nerad, T. A., Anderson, O. R., & Reysenbach, A.-L. (2006). *Vannella epipetala n. sp.* isolated from the leaf surface of *Spondias mombin* (Anacardiaceae) growing in the dry forest of Costa Rica. *Journal of Eukaryotic Microbiology*, *53*(6), 522–530.

American Optometric Association. (2003). Facts and stats. Retrieved September 2, 2017, from https://www.aoa.org/patients-and-public/caring-for-your-vision/contact-lenses/facts-and-stats

Anand, C. M., Skinner, A. R., Malic, A., & Kurtz, J. B. (1983). Interaction of *L. pneumophilia* and a free living amoeba (*Acanthamoeba palestinensis*). *The Journal of Hygiene*, *91*(2), 167–178. https://doi.org/10.1017/S0022172400060174

Anderson, I. J., Watkins, R. F., Samuelson, J., Spencer, D. F., Majoros, W. H., Gray, M. W., & Loftus, B. J. (2005). Gene discovery in the *Acanthamoeba castellanii* genome. *Protist*, *156*(2), 203–214. https://doi.org/10.1016/j.protis.2005.04.001

Anderson, O. R., Nerad, T. A., & Cole, J. C. (2003). *Platyamoeba nucleolilateralis n. sp.* from the Chesapeake Bay Region. *The Journal of Eukaryotic Microbiology*, *50*(1), 57–60.

Andrews, S. (n.d.). FastQC A quality control tool for high throughput sequence data. Retrieved from http://www.bioinformatics.babraham.ac.uk/projects/fastqc

Aqeel, Y., Siddiqui, R., Iftikhar, H., & Khan, N. A. (2013). The effect of different environmental conditions on the encystation of *Acanthamoeba castellanii* belonging to the T4 genotype. *Experimental Parasitology*, *135*(1), 30–35. https://doi.org/10.1016/j.exppara.2013.05.017

Aqeel, Y., Siddiqui, R., Manan, Z., & Khan, N. A. (2015). The role of G protein coupled receptor-mediated signaling in the biological properties of *Acanthamoeba castellanii* of the T4 genotype. *Microbial Pathogenesis*, *81*, 22–27. https://doi.org/10.1016/j.micpath.2015.03.006

Astorga, B., Lorenzo-Morales, J., Martín-Navarro, C. M., Alarcõn, V., Moreno, J., González, A. C., … Valladares, B. (2011). *Acanthamoeba* belonging to T3, T4, and T11: genotypes isolated from air-conditioning units in Santiago, Chile. *Journal of Eukaryotic Microbiology*, *58*(6), 542–544. https://doi.org/10.1111/j.1550-7408.2011.00584.x

Auran, J. D., Starr, M. B., & Jakobiec, F. A. (1987). *Acanthamoeba* keratitis: a review of the literature. *Cornea*, *6*(1), 2–26.

Aurrecoechea, C., Barreto, A., Brestelli, J., Brunk, B. P., Caler, E. V., Fischer, S., … Wang, H. (2011). AmoebaDB and MicrosporidiaDB: functional genomic resources for Amoebozoa and Microsporidia species. *Nucleic Acids Research*, *39*(SUPPL. 1), 612–619. https://doi.org/10.1093/nar/gkq1006

Bagheri, H. R., Shafiei, R., Shafiei, F., & Sajjadi, S. A. (2010). Isolation of *Acanthamoeba spp.* from drinking waters in several hospitals of Iran. *Iranian Journal of Parasitology*, *5*(2), 19–25.

Baines, I. C., Brzeska, H., & Korn, E. D. (1992). Differential localization of *Acanthamoeba* myosin-I isoforms. *The Journal of Cell Biology*, *119*(5), 1193–1203.

Baldauf, S. L. (2008). An overview of the phylogeny and diversity of eukaryotes. *Journal of*

Systematics and Evolution, *46*(3), 263–273. https://doi.org/10.3724/SP.J.1002.2008.08060

Bamforth, S. S. (2008). Protozoa of biological soil crusts of a cool desert in Utah. *Journal of Arid Environments*, *72*(5), 722–729. https://doi.org/10.1016/j.jaridenv.2007.08.007

Baneyx, F. (1999). Recombinant protein expression in *Escherichia coli*. *Current Opinion in Biotechnology*.

Bapteste, E., Brinkmann, H., Lee, J., Moore, D., Sensen, C., Gordon, P., … Philippe, H. (2002). The analysis of 100 genes supports the grouping of three highly divergent amoebae: *Dictyostelium, Entamoeba,* and *Mastigamoeba*. *Proceedings of the National Academy of Sciences*, *99*(3), 1414–1419. https://doi.org/10.1073/pnas.032662799

Bardelang, P., Vankemmelbeke, M., Zhang, Y., Jarvis, H., Antoniadou, E., Rochette, S., … James, R. (2009). Design of a polypeptide FRET substrate that facilitates study of the antimicrobial protease lysostaphin. *The Biochemical Journal*, *418*(3), 615–624. https://doi.org/10.1042/BJ20081765

Barker, J., Humphrey, T. J., & Brown, M. W. R. (1999). Survival of *Escherichia coli* 0157 in a soil protozoan: implications for disease. *FEMS Microbiology Letters*, *173*(2), 291–295. Retrieved from http://femsle.oxfordjournals.org/content/173/2/291.abstract

Barker, J., Lambert, P. a, & Brown, M. R. (1993). Influence of intra-amoebic and other growth conditions on the surface properties of *Legionella pneumophila*. *Infection and Immunity*, *61*(8), 3503–3510. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=281029&tool=pmcentrez&rendertype=abstract

Barker, J., Scaife, H., & Brown, M. R. W. (1995). Intraphagocytic growth induces an antibiotic-resistant phenotype of *Legionella pneumophila*. *Antimicrobial Agents and Chemotherapy*, *39*(12), 2684–2688. https://doi.org/10.1128/AAC.39.12.2684.Updated

Bayer-Santos, E., Aguilar-Bonavides, C., Rodrigues, S. P., Cordero, E. M., Marques, A. F., Varela-Ramirez, A., … Almeida, I. C. (2013). Proteomic analysis of *Trypanosoma cruzi* secretome: characterization of two populations of extracellular vesicles and soluble proteins. *Journal of Proteome Research*, *12*(2), 883–897. https://doi.org/10.1021/pr300947g

Behera, H. S., & Satpathy, G. (2016). Characterisation and expression analysis of trophozoite and cyst proteins of *Acanthamoeba spp.* isolated from *Acanthamoeba* keratitis (AK) patient. *Molecular and Biochemical Parasitology*, *205*(1), 29–34. https://doi.org/https://doi.org/10.1016/j.molbiopara.2016.03.009

Behniafar, H., Niyyati, M., Lasjerdi, Z., & Dodangeh, S. (2015). High occurrence of potentially pathogenic free-living amoebae in water bodies of Kaleybar and Khodaafarin, East Azerbaijan province. *Current World Environment*, *10*(1), 727–731. https://doi.org/10.12944/CWE.10.Special-Issue1.87

Berger, D., & Mannheim, W. (1970). Anreicherung und charakterisierung eines bakteriolytischen Amöbenenzyms. *Zeitschrift Fur Naturforschung - Section B Journal of Chemical Sciences*, *25*(9), 1002–1010. https://doi.org/10.1515/znb-1970-0919

Biddick, C. J., Rogers, L. H., & Brown, T. J. (1984). Viability of pathogenic and nonpathogenic free-living amoebae in long-term storage at a range of temperatures. *Applied and Environmental Microbiology*, *48*(4), 859–860.

Birkeland, S. R., Preheim, S. P., Davids, B. J., Cipriano, M. J., Palm, D., Reiner, D. S., … McArthur, A. G. (2010). Transcriptome analyses of the *Giardia lamblia* life cycle. *Molecular and Biochemical Parasitology*, *174*(1), 62–65. https://doi.org/10.1016/j.molbiopara.2010.05.010

Blackman, H. J., Rao, N. A., Lemp, M. A., & Visvesvara, G. S. (1984). *Acanthamoeba* keratitis successfully treated with penetrating keratoplasty: suggested immunogenic mechanisms of action. *Cornea*, *3*(2), 125–130. Retrieved from http://journals.lww.com/corneajrnl/Fulltext/1984/02000/Acanthamoeba_Keratitis_Successfully_Treated_with.9.aspx

Blanton, W. E., & Villemez, C. L. (1978). Molecular size and chain length distribution in *Acanthamoeba* cellulose. *The Journal of Protozoology*, *25*(2), 264–267.

Blume, J. E., & Ennis, H. L. (1991). A *Dictyostelium discoideum* cellulase is a member of a spore germination-specific gene family. *Journal of Biological Chemistry*, *266*(23), 15432–15437.

Bohnert, H. J., & Herrmann, R. G. (1974). The genomic complexity of *Acanthamoeba castellanii* mitochondrial DNA. *European Journal of Biochemistry*, *50*, 83–90.

Booton, G. C., Kelly, D. J., Chu, Y. W., Seal, D. V., Houang, E., Lam, D. S. C., … Fuerst, P. A. (2002). 18S ribosomal DNA typing and tracking of *Acanthamoeba* species isolates from corneal scrape specimens, contact lenses, lens cases, and home water supplies of *Acanthamoeba* keratitis patients in Hong Kong. *Journal of Clinical Microbiology*, *40*(5), 1621–1625. https://doi.org/10.1128/JCM.40.5.1621-1625.2002

Bovee, E. C. (1965). An emendation of the ameba genus *Flabellula* and a description of *Vannella gen. nov. Transactions of the American Microscopical Society*, *84*(2), 217–227.

Bowers, B., & Korn, E. D. (1968). The fine structure of *Acanthamoeba castellanii*. *The Journal of Cell Biology*, *39*(1), 95–111.

Bowers, B., & Korn, E. D. (1974). Localization of lipophosphonoglycan on both sides of *Acanthamoeba* plasma membrane. *Journal of Cell Biology*, *62*(2), 533–540. https://doi.org/10.1083/jcb.62.2.533

Bowers, B., & Olszewski, T. E. (1972). Pinocytosis in *Acanthamoeba castellanii*: kinetics and morphology. *Journal of Cell Biology*, *53*(3), 681–694. https://doi.org/10.1083/jcb.53.3.681

Bowers, B., & Olszewski, T. E. (1983). *Acanthamoeba* discriminates internally between digestible and indigestible particles. *Journal of Cell Biology*, *97*(2), 317–322. https://doi.org/10.1083/jcb.97.2.317

Boyer, M., Yutin, N., Pagnier, I., Barrassi, L., Fournous, G., Espinosa, L., … Raoult, D. (2009). Giant *Marseillevirus* highlights the role of amoebae as a melting pot in emergence of chimeric microorganisms. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(51), 21848–21853. https://doi.org/10.1073/pnas.0911354106

Bradley, S. G., & Marciano-Cabral, F. (1996). Diversity of free-living “naked” amoeboid organisms. *Journal of Industrial Microbiology*, *17*(3–4), 314–321. https://doi.org/10.1007/BF01574706

Brindley, N., Matin, A., & Khan, N. A. (2009). *Acanthamoeba castellanii*: high antibody prevalence in racially and ethnically diverse populations. *Experimental Parasitology*, *121*(3), 254–256. https://doi.org/10.1016/j.exppara.2008.11.009

Brown, M. W., & Silberman, J. D. (2013). The non-dictyostelid sorocarpic amoebae. In M. Romeralo, S. Baldauf, & R. Escalante (Eds.), *Dictyostelids: Evolution, Genomics and Cell Biology* (pp. 219–242). Berlin, Heidelberg: Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-642-38487-5_12

Brown, M. W., Silberman, J. D., & Spiegel, F. W. (2012). A contemporary evaluation of the acrasids (Acrasidae, Heterolobosea, Excavata). *European Journal of Protistology*, *48*(2), 103–123. https://doi.org/10.1016/j.ejop.2011.10.001

Brown, S., & De Jonckheere, J. F. (1999). A reevaluation of the amoeba genus *Vahlkampfia* based on SSUrDNA sequences. *European Journal of Protistology*, *35*(1), 49–54. https://doi.org/10.1016/S0932-4739(99)80021-2

Brown S, & Smirnov AV. (2004). Diversity of gymnamoebae in grassland soil in Southern Scotland. *Protistology*, *3*(3), 191–195.

Bui, T. H., Cavanagh, H. D., & Robertson, D. M. (2010). Patient compliance during contact lens wear: perceptions awareness and behavior. *Eye Contact Lens*, *36*(6), 334–339. https://doi.org/10.1007/s12020-009-9266-z.A

Byers, T. J. (1986). Molecular biology of DNA in *Acanthamoeba, Amoeba, Entamoeba*, and *Naegleria*. *International Review of Cytology*, *99*, 311–341.

Byers, T. J., Hugo, E. R., & Stewart, V. J. (1990). Genes of *Acathamoeba*: DNA, RNA and protein sequences (A review). *Journal of Protozoology*, *37*(4), 17S–25S.

Cabello-Vílchez, A. M., Martín-Navarro, C. M., López-Arencibia, A., Reyes-Batlle, M., Sifaoui, I.,

Valladares, B., … Lorenzo-Morales, J. (2014). Voriconazole as a first-line treatment against potentially pathogenic *Acanthamoeba* strains from Peru. *Parasitology Research*, *113*(2), 755–759. https://doi.org/10.1007/s00436-013-3705-8

Cachia Markham, M., & Mercieca, F. (2012). Bilateral *Acanthamoeba* keratitis. *The Synapse*, (1), 14–15. https://doi.org/10.1097/ICO.0b013e3181861bf9

Cammaroto, G., Astorga, F. J., Navarro, A., Olive, T., & Pumarola, F. (2015). *Acanthamoeba* rhinosinusitis: a paediatric case report and a review of the literature. *International Journal of Pediatric Otorhinolaryngology Extra*, *10*(3), 70–73. https://doi.org/10.1016/j.pedex.2015.07.002

Campanelli, D., Melchior, M., Fu, Y. P., Nakata, M., Shuman, H., Nathan, C., & Gabay, J. E. (1990). Cloning of cDNA for proteinase-3 - a serine protease, antibiotic, and autoantigen from human neutrophils. *Journal of Experimental Medicine*, *172*(6), 1709–1715. https://doi.org/Doi 10.1084/Jem.172.6.1709

Cao, Z., Jefferson, D. M., & Panjwani, N. (1998). Role of carbohydrate-mediated adherence in cytopathogenic mechanisms of *Acanthamoeba*. *Journal of Biological Chemistry*, *273*(25), 15838–15845. https://doi.org/10.1074/jbc.273.25.15838

Carnt, N., Connor, S., Parayoganathan, V., & Dart, J. (2015). The quality of life of contact lens wearers experiencing *Acanthamoeba* keratitis. *Contact Lens and Anterior Eye*, *38*(2015), e9. https://doi.org/10.1016/j.clae.2014.11.108

Carruthers, V. B., Håkansson, S., Olivia, K., Sibley, L. D., & Giddings, O. K. (2000). *Toxoplasma gondii* uses sulfated proteoglycans for substrate and host cell attachment. *Infection and Immunity*, *68*(7), 4005–4011. https://doi.org/10.1128/IAI.68.7.4005-4011.2000.Updated

Carter, R. F. (1968). Primary amoebic meningo-encephalitis: clinical, pathological and epidemiological features of six fatal cases. *The Journal of Pathology and Bacteriology*, *96*(1), 1–25. https://doi.org/10.1002/path.1700960102

Carter, R. F. (1970). Description of a *Naegleria sp.* isolated from two cases of primary amoebic meningo-encephalitis, and of the experimental pathological changes induced by it. *The Journal of Pathology*, *100*(4), 217–244. https://doi.org/10.1002/path.1711000402

Castellani, A. (1930). An amoeba found in culture of yeast: preliminary note. *Journal of Tropical Medicine and Hygiene*, *33*, 160.

Cavalier-Smith, T., & Chao, E. E. (2003). Phylogeny and classification of phylum Cercozoa (Protozoa). *Protist*, *154*(3–4), 341–358. https://doi.org/10.1078/143446103322454112

Cavalier-Smith, T., Chao, E. E., & Lewis, R. (2016). 187-gene phylogeny of protozoan phylum Amoebozoa reveals a new class (Cutosea) of deep-branching, ultrastructurally unique, enveloped marine Lobosa and clarifies amoeba evolution. *Molecular Phylogenetics and Evolution*, *99*, 275–296. https://doi.org/10.1016/j.ympev.2016.03.023

Chagla, A. H., & Griffiths, A. J. (1974). Growth and encystation of *Acanthamoeba castellanii*. *Journal of General Microbiology*, *85*(1974), 139–145.

Chappell, C. L., Wright, J. a, Coletta, M., & Newsome, A. L. (2001). Standardized method of measuring *Acanthamoeba* antibodies in sera from healthy human subjects. *Clinical and Diagnostic Laboratory Immunology*, *8*(4), 724–730. https://doi.org/10.1128/CDLI.8.4.724

Chau, T. M., & Muller, H. E. (1990). Ingested *Listeria monocytogenes* survive and multiply in protozoa. *The Pathological Society of Great Britain and Ireland*, *33*(March), 51–54. Retrieved from http://doi.wiley.com/10.1111/j.1365-2672.1990.tb02904.x

Chavatte, N., Lambrecht, E., Van Damme, I., Sabbe, K., & Houf, K. (2016). Abundance, diversity and community composition of free-living protozoa on vegetable sprouts. *Food Microbiology*, *55*, 55–63. https://doi.org/10.1016/j.fm.2015.11.013

Chávez-Munguía, B., Omaña-Molina, M., González-Lázaro, M., González-Robles, A., Cedillo-Rivera, R., Bonilla, P., & Martínez-Palomo, A. (2007). Ultrastructure of cyst differentiation in parasitic protozoa. *Parasitology Research*, *100*(6), 1169–1175. https://doi.org/10.1007/s00436-006-0447-x

Chávez-Munguía, B., Salazar-Villatoro, L., Lagunes-Guillén, A., Omaña-Molina, M., Espinosa-

Cantellano, M., & Martínez-Palomo, A. (2013). *Acanthamoeba castellanii* cysts: new ultrastructural findings. *Parasitology Research*, *112*(3), 1125–1130. https://doi.org/10.1007/s00436-012-3261-7

Chen, L., Orfeo, T., Gilmartin, G., & Bateman, E. (2004). Mechanism of cyst specific protein 21 mRNA induction during *Acanthamoeba* differentiation. *Biochimica et Biophysica Acta*, *1691*(1), 23–31. https://doi.org/10.1016/j.bbamcr.2003.11.005

Chitrampalam, P., Goldberg, N., & Olsen, M. W. (2015). *Labyrinthula* species associated with turfgrasses in Arizona and New Mexico. *European Journal of Plant Pathology*, *143*(3), 485–493. https://doi.org/10.1007/s10658-015-0701-0

Cho, J. H., Na, B. K., Kim, T. S., & Song, C. Y. (2000). Purification and characterization of an extracellular serine proteinase from *Acanthamoeba castellanii*. *IUBMB Life*, *50*(September), 209–214.

Chung, D. I., Yu, H. S., Hwang, M. Y., Kim, T. H., Kim, T. O., Yun, H. C., & Kong, H. H. (1998). Subgenus classification of *Acanthamoeba* by riboprinting. *The Korean Journal of Parasitology*.

Chung, W. O., Hansen, S. R., Rao, D., & Dale, B. A. (2004). Protease-activated receptor signaling increases epithelial antimicrobial peptide expression. *The Journal of Immunology*, *173*(8), 5165–5170. https://doi.org/10.4049/jimmunol.173.8.5165

Cirillo, J. D., Cirillo, S. L., Yan, L., Bermudez, L. E., Falkow, S., & Tompkins, L. S. (1999). Intracellular growth in *Acanthamoeba castellanii* affects monocyte entry mechanisms and enhances virulence of *Legionella pneumophila*. *Infection and Immunity*, *67*(9), 4427–4434. Retrieved from http://iai.asm.org/content/67/9/4427.abstract

Cirillo, J. D., Falkow, S., Tompkins, L., & Bermudez, L. (1997). Interaction of *Mycobacterium avium* with environmental amoebae enhances virulence. *Infection and Immunity*, *65*(9), 3759–3767. Retrieved from http://iai.asm.org/cgi/content/long/65/9/3759

Cirillo, J. D., Falkow, S., & Tompkins, L. S. (1994). Growth of *Legionella pneumophila* in *Acanthamoeba castellanii* enhances invasion. *Infection and Immunity*, *62*(8), 3254–3261.

Clarholm, M. (1981). Protozoan grazing of bacteria in soil - Impact and importance. *Microbial Ecology*, *7*(4), 343–350. Retrieved from http://www.springerlink.com/content/7wxh772p46205223/fulltext.pdf

Clarholm, M. (2002). Bacteria and protozoa as integral components of the forest ecosystem - Their role in creating a naturally varied soil fertility. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology*, *81*, 309–318. https://doi.org/10.1023/A:1020543424098

Clark, C. G. (1990). Genome structure and evolution of *Naegleria* and its relatives. *Journal of Protozoology*, *37*(4), 2S–6S.

Clark, C. G., & Cross, G. A. (1987). rRNA genes of *Naegleria gruberi* are carried exclusively on a 14-kilobase-pair plasmid. *Molecular and Cellular Biology*, *7*(9), 3027–3031. https://doi.org/10.1128/mcb.7.9.3027

Clark, K., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., & Sayers, E. W. (2016). GenBank. *Nucleic Acids Research*, *44*(D1), D67–D72. https://doi.org/10.1093/nar/gkv1276

Clarke, D. W., Alizadeh, H., & Niederkorn, J. Y. (2005). Failure of *Acanthamoeba castellanii* to produce intraocular infections. *Investigative Ophthalmology and Visual Science*, *46*(7), 2472–2478. https://doi.org/10.1167/iovs.05-0140

Clarke, D. W., & Niederkorn, J. Y. (2006). The pathophysiology of *Acanthamoeba* keratitis. *Trends in Parasitology*, *22*(4), 175–180. https://doi.org/10.1016/j.pt.2006.02.004

Clarke, M., Lohan, A. J., Liu, B., Lagkouvardos, I., Roy, S., Zafar, N., … Loftus, B. J. (2013). Genome of *Acanthamoeba castellanii* highlights extensive lateral gene transfer and early evolution of tyrosine kinase signaling. *Genome Biology*, *14*(2), R11. https://doi.org/10.1186/gb-2013-14-2-r11

Colson, P., Yutin, N., Shabalina, S. A., Robert, C., Fournous, G., La Scola, B., … Koonin, E. V. (2011). Viruses with more than 1,000 genes: mamavirus, a new *Acanthamoeba polyphaga*

mimivirus strain, and reannotation of mimivirus genes. *Genome Biology and Evolution*, *3*(0), 737–742. https://doi.org/10.1093/gbe/evr048

Conza, L., Pagani, S. C., & Gaia, V. (2013). Presence of *Legionella* and free-living amoebae in composts and bioaerosols from composting facilities. *PLoS ONE*, *8*(7). https://doi.org/10.1371/journal.pone.0068244

Cope, J. R., Collier, S. A., Rao, M. M., Chalmers, R., Mitchell, G. L., Richdale, K., … Beach, M. J. (2015). Contact lens wearer demographics and risk behaviors for contact lens-related eye infections--United States, 2014. *Morbidity and Mortality Weekly Report*, *64*(32), 865–870. https://doi.org/10.15585/mmwr.mm6432a2

Cordingley, J. S., Wills, R. A., & Clarence L. Villemez. (1996). Osmolarity is an independent trigger of *Acanthamoeba castellanii* differentiation. *Journal of Cellular Biochemistry*, *171*(1), 167–171.

Corrêa, A. P. F., Daroit, D. J., Coelho, J., Meira, S. M., Lopes, F. C., Segalin, J., … Brandelli, A. (2011). Antioxidant, antihypertensive and antimicrobial properties of ovine milk caseinate hydrolyzed with a microbial protease. *Journal of the Science of Food and Agriculture*, *91*(12), 2247–2254. https://doi.org/10.1002/jsfa.4446

Corsaro, D., Köhsler, M., Montalbano Di Filippo, M., Venditti, D., Monno, R., Di Cave, D., … Walochnik, J. (2017). Update on *Acanthamoeba jacobsi* genotype T15, including full-length 18S rDNA molecular phylogeny. *Parasitology Research*, *116*(4), 1273–1284. https://doi.org/10.1007/s00436-017-5406-1

Corsaro, D., Michel, R., Walochnik, J., Müller, K. D., & Greub, G. (2010). *Saccamoeba lacustris, sp. nov.* (Amoebozoa: Lobosea: Hartmannellidae), a new lobose amoeba, parasitized by the novel chlamydia *'Candidatus* Metachlamydia lacustris' (Chlamydiae: *Parachlamydiaceae*). *European Journal of Protistology*, *46*(2), 86–95. https://doi.org/10.1016/j.ejop.2009.11.002

Corsaro, D., Müller, K. D., Wingender, J., & Michel, R. (2013). *"Candidatus Mesochlamydia elodeae"* (Chlamydiae: Parachlamydiaceae), a novel chlamydia parasite of free-living amoebae. *Parasitology Research*, *112*(2), 829–838. https://doi.org/10.1007/s00436-012-3213-2

Corsaro, D., & Venditti, D. (2011). More *Acanthamoeba* genotypes: limits to the use of rDNA fragments to describe new genotypes. *Acta Protozoologica*, *50*(1), 49–54. https://doi.org/10.1111/j.1755-3768.2011.02269.x

Corsaro, D., & Venditti, D. (2017). An apparent *Acanthamoeba* genotype is the product of a chimeric 18S rDNA artifact. *Parasitology Research*, 1–7.

Corsaro, D., Walochnik, J., Köhsler, M., & Rott, M. B. (2015). *Acanthamoeba* misidentification and multiple labels: redefining genotypes T16, T19, and T20 and proposal for *Acanthamoeba micheli sp. nov.* (genotype T19). *Parasitology Research*, *114*(7), 2481–2490. https://doi.org/10.1007/s00436-015-4445-8

Corsaro, D., Walochnik, J., Venditti, D., Steinmann, J., Müller, K. D., & Michel, R. (2014). Microsporidia-like parasites of amoebae belong to the early fungal lineage Rozellomycota. *Parasitology Research*, *113*(5), 1909–1918. https://doi.org/10.1007/s00436-014-3838-4

Cosson, P., & Lima, W. C. (2014). Intracellular killing of bacteria: Is *Dictyostelium* a model macrophage or an alien? *Cellular Microbiology*, *16*(6), 816–823. https://doi.org/10.1111/cmi.12291

Costas, M., & Griffiths, A. J. (1980). The suitability of starch-gel electrophoresis of esterases and acid- phosphatases for the study of *Acanthamoeba* taxonomy. *Archiv Fur Protistenkunde*, *123*(3), 272–279. https://doi.org/10.1016/S0003-9365(80)80012-1

Costas, M., & Griffiths, A. J. (1985). Enzyme composition and the taxonomy of *Acanthamoeba*. *The Journal of Protozoology*, *32*(4), 604–607. https://doi.org/10.1111/j.1550-7408.1985.tb03086.x

Coulon, C., Dechamps, N., Meylheuc, T., Collignon, A., McDonnell, G., & Thomas, V. (2012). The effect of in vitro growth conditions on the resistance of *Acanthamoeba* cysts. *The Journal of Eukaryotic Microbiology*, *59*(3), 198–205. https://doi.org/10.1111/j.1550-7408.2012.00612.x

Crary, M. J., Narayanan, S., Visvesvara, G., Joslin, C., Tu, E., Panjwani, N., … Fuerst, P. (2011). Genotyping *Acanthamoeba* keratitis (AK) infections: cytochrome oxidase I (COI) barcoding and phylogenetic analysis. *Investigative Ophthalmology & Visual Science*, *52*(14), 5832. Retrieved from http://dx.doi.org/

Creasey, A. M., Staalsoe, T., Raza, A., Arnot, D. E., & Rowe, J. A. (2003). Nonspecific immunoglobulin M binding and chondroitin sulfate A binding are linked phenotypes of *Plasmodium falciparum* isolates implicated in malaria during pregnancy. *Infection and Immunity*, *71*(8), 4767–4771. https://doi.org/10.1128/IAI.71.8.4767-4771.2003

Culbertson, C. G., Smith, J. W., & Minner, J. R. (1958). *Acanthamoeba*: observations on animal pathogenicity. *Science*, *127*(3313), 1506. https://doi.org/10.1126/science.127.3313.1506

Cursiefen, C. (2007). Immune privilege and angiogenic privilege of the cornea. *Chemical Immunology and Allergy*, *92*, 50–57. https://doi.org/10.1159/000099253

Cursons, R. T. M., Brown, T. J., Keys, E. A., Moriarty, K. M., & Till, D. (1980). Immunity to pathogenic free-living amoebae: role of cell-mediated immunity. *Infection and Immunity*, *29*(2), 408–410.

Da Lage, J. L., Danchin, E. G. J., & Casane, D. (2007). Where do animal α-amylases come from? An interkingdom trip. *FEBS Letters*, *581*(21), 3927–3935. https://doi.org/10.1016/j.febslet.2007.07.019

da Rocha-Azevedo, B., Tanowitz, H. B., & Marciano-Cabral, F. (2009). Diagnosis of infections caused by pathogenic free-living amoebae. *Interdisciplinary Perspectives on Infectious Diseases*, *2009*, 1–14. https://doi.org/10.1155/2009/251406

De Cádiz, A. E., Jeelani, G., Nakada-Tsukui, K., Caler, E., & Nozaki, T. (2013). Transcriptome analysis of encystation in *Entamoeba invadens*. *PLoS ONE*, *8*(9), e74840. https://doi.org/10.1371/journal.pone.0074840

De Jonckheere, Johan F., Jun Murase, F. R. O. (2011). A new thermophilic heterolobosean amoeba, *Fumarolamoeba ceburocoi, gen. nov., sp. nov.*, isolated near a fumarole at a volcano in Mexico. *Acta Protozoologica*, *50*, 43–50.

De Jonckheere, J. F. (1980). Growth charactertistics, cytopathic effect in cell culture, and virulence in mice of 36 type strains belonging to 19 different *Acanthamoeba spp. Applied and Environmental Microbiology*, *39*(4), 681–685.

de Obeso Fernandez del Valle, A., Lorenzo-Morales, J., & Maciver, S. K. (2017). Leptomyxa valladaresi n. sp. (Amoebozoa, Tubulinea, Leptomyxida), from Mount Teide, Tenerife, Spain. *Experimental Parasitology*, *183*, 85–91. https://doi.org/10.1016/j.exppara.2017.09.017

de Obeso Fernandez del Valle, A., & Maciver, S. (2017). Allovahlkampfia minuta nov. sp., (Acrasidae, Heterolobosea, Excavata) a new soil amoeba at the boundary of the acrasid cellular slime moulds. *Acta Protozoologica*, *56*, 181–189.

Deichmann, U., Jantzen, H., & Deutschland, B. (1977). The cellulase enzyme system during growth and development of *Acanthamoeba castellanii*. *Archives of Microbiology*, *113*(3), 309–313.

Detke, S., & Paule, M. R. (1975). DNA-dependent RNA polymerases from *Acanthamoeba castellanii*: properties and levels of activity during encystment. *Biochimica et Biophysica Acta*, *383*, 67–77.

Dey, R., Hoffman, P. S., & Glomski, I. J. (2012). Germination and amplification of anthrax spores by soil-dwelling amoebas. *Applied and Environmental Microbiology*, *78*(22), 8075–8081. https://doi.org/10.1128/AEM.02034-12

Dickson, J. M., Zetler, P. J., Walker, B., & Javer, A. R. (2009). *Acanthamoeba* rhinosinusitis. *Journal of Otolaryngology - Head and Neck Surgery*, *38*(3), 87–91. https://doi.org/10.2310/7070.2009.080157

Doberstein, S. K., Baines, I. C., Wiegand, G., Korn, E. D., & Pollard, T. D. (1993). Inhibition of contractile vacuole function in-vivo by antibodies against myosin-I. *Nature*, *365*(6449), 841–843. https://doi.org/10.1038/365841a0

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., … Gingeras, T. R. (2013). STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics*, *29*(1), 15–21. https://doi.org/10.1093/bioinformatics/bts635

Douglas, M. (1930). Notes on the classification of the amoeba found by Castellani in cultures of a yeast-like fungus. *Journal of Tropical Medicine and Hygiene*, *33*, 258–259.

Doumas, S., & Kolokotronis, A. (2005). Anti-inflammatory and antimicrobial roles of secretory leukocyte protease inhibitor. *Infection and Immunity*, *73*(3), 1271–1274. https://doi.org/10.1128/IAI.73.3.1271

Drozanski, W. (1969). Bacteriolytic spectrum of the enzyme produced by *Acanthamoeba castellanii*. *Acta Microbiologica Polonica*, (3/4), 169–174.

Drozanski, W. (1971). Purification and properties of bacteriolytic enzyme produced by *Acanthamoeba castellanii*. *Acta Microbiologica Polonica*, *IV*(XXI), 33–52.

Drozański, W. (1969). Bacteriolytic enzyme produced by *Acanthamoeba castellanii*. *Acta Microbiologica Polonica*, *1*(3), 155.

Drozański, W. (1978). Two forms of bacteriolytic endo-N-acetylmuramidase in *Acanthamoeba castellanii*. *Acta Biochimica Polonica*, *25*(3), 229–238.

Drozanski, W. J. (1991). *Sarcobium zyticum gen . nov ., sp . nov .*, an obligate intracellular bacterial parasite of small free-living amoebae. *International Journal*, *41*(1), 82–87.

Dudley, R., Jarroll, E. L., & Khan, N. A. (2009). Carbohydrate analysis of *Acanthamoeba castellanii*. *Experimental Parasitology*, *122*(4), 338–343. https://doi.org/10.1016/j.exppara.2009.04.009

Dyková, I., Boháčová, L., Fiala, I., Macháčková, B., Pecková, H., & Dvořáková, H. (2005). Amoebae of the genera *Vannella* Bovee, 1965 and *Platyamoeba* Page, 1969 isolated from fish and their phylogeny inferred from SSU rRNA gene and ITS sequences. *European Journal of Protistology*, *41*(3), 219–230. https://doi.org/10.1016/j.ejop.2005.05.004

Dyková, I., Lom, J., Schroeder-diedrich, J. M., Booton, G. C., & Byers, T. J. (1999). *Acanthamoeba* strains isolated from organs of freshwater fishes. *The Journal of Parasitology*, *85*(6), 1106–1113.

Ehrenkaufer, G. M., Haque, R., Hackney, J. A., Eichinger, D. J., & Singh, U. (2007). Identification of developmentally regulated genes in *Entamoeba histolytica*: insights into mechanisms of stage conversion in a protozoan parasite. *Cellular Microbiology*, *9*(6), 1426–1444. https://doi.org/10.1111/j.1462-5822.2006.00882.x

El-Etr, S. H., Margolis, J. J., Monack, D., Robison, R. A., Cohen, M., Moore, E., & Rasley, A. (2009). *Francisella tularensis* type A strains cause the rapid encystment of *Acanthamoeba castellanii* and survive in amoebal cysts for three weeks postinfection. *Applied and Environmental Microbiology*, *75*(23), 7488–7500. https://doi.org/10.1128/AEM.01829-09

Elliott, E. T., & Coleman, D. C. (1977). Soil protozoan dynamics in a shortgrass prairie. *Soil Biology and Biochemistry*, *9*(2), 113–118. https://doi.org/10.1016/0038-0717(77)90046-3

Essig, A., Heinemann, M., Simnacher, U., & Marre, R. (1997). Infection of *Acanthamoeba castellanii* by *Chlamydia pneumoniae*. *Applied and Environmental Microbiology*, *63*(4), 1396–1399.

Faye, T., Brede, D. A., Langsrud, T., Nes, I. F., & Holo, H. (2004). Prevalence of the genes encoding propionicin T1 and protease-activated antimicrobial peptide and their expression in classical *Propionibacteria*. *Applied and Environmental Microbiology*, *70*(4), 2240–2244. https://doi.org/10.1128/AEM.70.4.2240-2244.2004

Fernández-Castané, A., Vine, C. E., Caminal, G., & López-Santín, J. (2012). Evidencing the role of lactose permease in IPTG uptake by *Escherichia coli* in fed-batch high cell density cultures. *Journal of Biotechnology*, *157*(3), 391–398. https://doi.org/10.1016/j.jbiotec.2011.12.007

Ferreira, G. A., Magliano, A. C. M., Pral, E. M. F., & Alfieri, S. C. (2009). Elastase secretion in *Acanthamoeba polyphaga*. *Acta Tropica*, *112*(2), 156–163. https://doi.org/10.1016/j.actatropica.2009.07.015

Ferrer-Bonet, M., & Ruiz-Trillo, I. (2017). *Capsaspora owczarzaki*. *Current Biology*, *27*(17), R829–R830. https://doi.org/10.1016/j.cub.2017.05.074

Fields, B. S., Benson, R. F., & Besser, R. E. (2002). *Legionella* and Legionnaires' disease: 25 years of investigation. *Clinical Microbiology Reviews*, *15*(3), 506–526. https://doi.org/10.1128/CMR.15.3.506-526.2002

Fleming, A., & Allison, V. D. (1922). Observations on a bacteriolytic substance ("lysozyme") found in secretions and tissues. *British Journal of Experimental Pathology*, *3*(5), 252.

Floyd, R., Abebe, E., Papert, A., & Blaxter, M. (2002). Molecular barcodes for soil nematode identification. *Molecular Ecology*, *11*(4), 839–850. https://doi.org/10.1046/j.1365-294X.2002.01485.x

Fraser, D. W., Tsai, T. R., Orenstein, W., Parkin, W. E., Beecham, H. J., Sharrar, R. G., … Brachman, P. S. (1977). Legionnaires' Disease. *The New England Journal of Medicine*, *297*(22), 1189–1197.

Fuerst, P. A., Booton, G. C., & Crary, M. (2015). Phylogenetic analysis and the evolution of the 18S rRNA gene typing system of Acanthamoeba. *Journal of Eukaryotic Microbiology*, *62*(1), 69–84. https://doi.org/10.1111/jeu.12186

Garate, M., Cao, Z., Bateman, E., & Panjwani, N. (2004). Cloning and characterization of a novel mannose-binding protein of *Acanthamoeba*. *Journal of Biological Chemistry*, *279*(28), 29849–29856. https://doi.org/10.1074/jbc.M402334200

Garate, M., Marchant, J., Cubillos, I., Cao, Z., Khan, N. A., & Panjwani, N. (2006). In vitro pathogenicity of *Acanthamoeba* is associated with the expression of the mannose-binding protein. *Investigative Ophthalmology and Visual Science*, *47*(3), 1056–1062. https://doi.org/10.1167/iovs.05-0477

Garduño-Vieyra, L., Gonzalez-Sanchez, C. R., & Hernandez-Da Mota, S. E. (2011). Ultraviolet-a light and riboflavin therapy for *Acanthamoeba* keratitis: a case report. *Case Reports in Ophthalmology*, *2*(2), 291–295. https://doi.org/10.1159/000331707

Gast, R. J., Ledee, D. R., Fuerst, P. A., & Byers, T. J. (1996). Subgenus systematics of *Acanthamoeba*: four nuclear 18S rDNA sequence types. *Journal of Eukaryotic Microbiology*, *43*(6), 498–504.

Gaze, W. H., Burroughs, N., Gallagher, M. P., & Wellington, E. M. H. (2003). Interactions between *Salmonella typhimurium* and *Acanthamoeba polyphaga*, and bbservation of a new mode of intracellular growth within contractile vacuoles. *Microbial Ecology*, *46*(3), 358–369. https://doi.org/10.1007/s00248-003-1001-3

Geisen, S., Bonkowski, M., Zhang, J., & De Jonckheere, J. F. (2015). Heterogeneity in the genus *Allovahlkampfia* and the description of the new genus *Parafumarolamoeba* (Vahlkampfiidae; Heterolobosea). *European Journal of Protistology*, *51*(4), 335–349. https://doi.org/10.1016/j.ejop.2015.05.003

Geisen, S., Fiore-Donno, A. M., Walochnik, J., & Bonkowski, M. (2014). Acanthamoeba everywhere: High diversity of *Acanthamoeba* in soils. *Parasitology Research*, *113*(9), 3151–3158. https://doi.org/10.1007/s00436-014-3976-8

Giessen, T. W., & Silver, P. A. (2016). Converting a natural protein compartment into a nanofactory for the size-constrained synthesis of antimicrobial silver nanoparticles. *ACS Synthetic Biology*, *5*(12), 1497–1504. https://doi.org/10.1021/acssynbio.6b00117

Glöckner, G., Eichinger, L., Szafranski, K., Pachebat, J. A., Bankier, A. T., Dear, P. H., … Winckler, T. (2002). Sequence and analysis of chromosome 2 of *Dictyostelium discoideum*. *Nature*, *418*, 79. Retrieved from http://dx.doi.org/10.1038/nature00847

Gomes de Amorim Santos, I., Scher, R., Rott, M. B., Menezes, L. R., Costa, E. V., Cavalcanti, S. C. de H., … Dolabella, S. S. (2016). Amebicidal activity of the essential oils of *Lippia spp.* (Verbenaceae) against *Acanthamoeba polyphaga* trophozoites. *Parasitology Research*, *115*(2), 535–540. https://doi.org/10.1007/s00436-015-4769-4

González-Robles, A., González-Lázaro, M., Omaña-Molina, M., & Martínez-Palomo, A. (2009). *Acanthamoeba castellanii*: endocytic structures involved in the ingestion of diverse target

elements. *Acta Protozoologica*, *48*(4), 329–334.

Gonzalez, M. M., Gould, E., Dickinson, G., Martinez, A. J., Visvesvara, G., Cleary, T. J., & Hensley, G. T. (1986). Acquired immunodeficiency syndrome associated with *Acanthamoeba* infection and other opportunistic organisms. *Archives of Pathology & Laboratory Medicine*, *110*(8), 749—751. Retrieved from http://europepmc.org/abstract/MED/3488048

Goodey, T. (1915). A preliminary communication on three new proteomyxan rhizopods from soil. *Arch. Protistenk*, *35*(80), 80–102.

Gordon, D. J., Eisenberg, E., & Korn, E. D. (1976). Characterization *Acanthamoeba* of cytoplasmic actin isolated from *Acanthamoeba castellanii* by a new method. *The Journal of Biological Chemistry*, (1), 4778–4786.

Gouy, M., Guindon, S., & Gascuel, O. (2010). SeaView version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Molecular Biology and Evolution*, *27*(2), 221–224. https://doi.org/10.1093/molbev/msp259

Grasso, C., & Lee, C. (2004). Combining partial order alignment and progressive multiple sequence alignment increases alignment speed and scalability to very large alignment problems. *Bioinformatics*, *20*(10), 1546–1556. https://doi.org/10.1093/bioinformatics/bth126

Greenwood, M., & Saunders, E. R. (1891). On the role of acid in protozoan digestion. *The Journal of Physiology*, *16*(5–6), 441–467.

Greub, G., & Raoult, D. (2004). Microorganisms resistant to free-living amoebae. *Clinical Microbiology Reviews*, *17*(2), 413–433. https://doi.org/10.1128/CMR.17.2.413-433.2004

Greub, G., & Scola, B. La. (2003). *Parachlamydia acanthamoeba* is endosymbiotic or lytic for *Acanthamoeba polyphaga* depending on the incubation temperature. *Annals of the New York Academy of Sciences*, *990*(00), 628–634. https://doi.org/10.1111/j.1749-6632.2003.tb07437.x

Griffiths, A. J., & Hughes, D. E. (1969). The physiology of encystment of *Hartmannella castellanii**. *The Journal of Protozoology*, *16*(1), 93–99. https://doi.org/10.1111/j.1550-7408.1969.tb02238.x

Guevara, M. G., Oliva, C. R., Huarte, M., & Daleo, G. R. (2002). An aspartic protease with antimicrobial activity is induced after infection and wounding in intercellular fluids of potato tubers. *European Journal of Plant Pathology*, *108*(2), 131–137. https://doi.org/10.1023/A:1015049629736

Guimaraes, A. J., Gomes, K. X., Cortines, J. R., Peralta, J. M., & Peralta, R. H. S. (2016). *Acanthamoeba spp.* as a universal host for pathogenic microorganisms: one bridge from environment to host virulence. *Microbiological Research*, *193*, 30–38. https://doi.org/10.1016/j.micres.2016.08.001

Haas, A. (2007). The phagosome: compartment with a license to kill. *Traffic*, *8*(4), 311–330. https://doi.org/10.1111/j.1600-0854.2006.00531.x

Hadas, E., Derda, M., & Cholewinski, M. (2017). Evaluation of the effectiveness of tea tree oil in treatment of *Acanthamoeba* infection. *Parasitology Research*, *116*(3), 997–1001. https://doi.org/10.1007/s00436-017-5377-2

Hadas, E., & Mazur, T. (1993). Proteolytic enzymes of pathogenic and non-pathogenic strains of *Acanthamoeba spp*. *Tropical Medicine and Parasitology*, *44*(3), 197–200. Retrieved from http://europepmc.org/abstract/MED/8256097

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*. Retrieved from http://jwbrown.mbio.ncsu.edu/JWB/papers/1999Hall1.pdf

Hammersmith, K. M. (2006). Diagnosis and management of *Acanthamoeba* keratitis. *Current Opinion in Ophthalmology*, *17*(4), 327–331. Retrieved from http://journals.lww.com/co-ophthalmology/Fulltext/2006/08000/Diagnosis_and_management_of_Acanthamoeba_keratitis.2.aspx

Harvey, R. A., Corenlisse, C. N., & Fisher, B. D. (2013). Protozoa. In Richard A. Harvey (Ed.),

Lippincott's Illustrated Reviews: Microbiology (Third, p. 448). Baltimore: Walters Kluwer. Retrieved from http://meded.lwwhealthlibrary.com.ezproxy.is.ed.ac.uk/content.aspx?sectionid=49552085&bookid=779#49554041

Hassan, A., Farouk, H., Hassanein, F., Abdul-Ghani, R., & Abdelhady, A. H. (2012). *Acanthamoeba* contamination of hemodialysis and dental units in Alexandria, Egypt: A neglected potential source of infection. *Journal of Infection and Public Health*, *5*(4), 304–310. https://doi.org/10.1016/j.jiph.2012.06.001

Hauer, G., Rogerson, A., & Anderson, O. R. (2001). *Platyamoeba pseudovannellida n. sp.*, a naked amoeba with wide salt tolerance isolated from the Salton Sea, California. *The Journal of Eukaryotic Microbiology*, *48*(6), 663–669.

Heffler, K. F., Eckhardt, T. J., Reboli, A. C., & Stieritz, D. (1996). *Acanthamoeba* endophthalmitis in acquired immunodeficiency syndrome. *American Journal of Ophthalmology*, *122*(4), 584–586. https://doi.org/10.1016/S0002-9394(14)72126-9

Helton, J., Loveless, M., & White, C. R. J. (1993). Cutaneous *Acanthamoeba* infection associated with leukocytoclastic vasculitis in an AIDS patient. *The American Journal of Dermatopathology*, *15*(2), 146–149. Retrieved from http://journals.lww.com/amjdermatopathology/Fulltext/1993/04000/Cutaneous_Acanthamoeba_Infection_Associated_with.8.aspx

Herbst, R., Ott, C., Jacobs, T., Marti, T., Marciano-cabral, F., & Leippe, M. (2002). Pore-forming polypeptides of the pathogenic protozoon *Naegleria fowleri*. *The Journal of Biological Chemistry*, *277*, 22353–22360. https://doi.org/10.1074/jbc.M201475200

Hettiarachchy, N. S., & Jones, I. G. (1974). Isolation and characterization of mitochondrial deoxyribonucleic acid of *Acanthamoeba castellanii*. *The Biochemical Journal*, *141*, 159–164.

Hewett, M. K., Robinson, B. S., Monis, P. T., & Saint, C. P. (2003). Identification of a new *Acanthamoeba* 18S rRNA gene sequence type, corresponding to the species *Acanthamoeba jacobsi* Sawyer, Nerad and Visvesvara, 1992 (Lobosea: Acanthamoebidae). *Acta Protozoologica*, *42*(4), 325–329.

Hibberd, D. J. (1971). Observations on the cytology and ultrastructure of *Chrysamoeba radians* klebs (Chrysophyceae). *British Phycological Journal*, *6*(2), 207–223. https://doi.org/10.1080/00071617100650231

Hiemstra, P. S. (2002). Novel roles of protease inhibitors in infection and inflammation. *Biochemical Society Transactions*, *30*(2), 116–120. https://doi.org/10.1042/

Hirukawa, Y., Nakato, H., Izumi, S., Tsuruhara, T., & Tomino, S. (1998). Structure and expression of a cyst specific protein of Acanthamoeba castellanii. *Biochimica et Biophysica Acta*, *1398*, 47–56. https://doi.org/10.1016/S0167-4781(98)00026-8

Hoffmann, R., Michel, R., Schmid, E. N., & Muller, K. D. (1998). Natural infection with microsporidian organisms (KW19) in Vannella spp. (Gymnamoebia) isolated from a domestic tap-water supply. *Parasitology Research*, *84*(2), 164–166. https://doi.org/10.1007/s004360050377

Hong, Y.-C., Lee, W.-M., Kong, H.-H., Jeong, H.-J., & Chung, D.-I. (2004). Molecular cloning and characterization of a cDNA encoding a laminin-binding protein (AhLBP) from *Acanthamoeba healyi*. *Experimental Parasitology*, *106*(3), 95–102.

Hong, Y. C., Kong, H. H., Ock, M. S., Kim, I. S., & Chung, D. I. (2000). Isolation and characterization of a cDNA encoding a subtilisin-like serine proteinase (AhSUB) from *Acanthamoeba healyi*. *Molecular and Biochemical Parasitology*, *111*, 441–446. https://doi.org/10.1016/S0166-6851(00)00326-1

Horn, M., Fritsche, T. R., Gautom, R. K., Schleifer, K.-H., & Wagner, M. (1999). Novel bacterial endosymbionts of *Acanthamoeba spp.* related to the *Paramecium caudatum* symbiont *Caedibacter caryophilus*. *Environmental Microbiology*, *1*(4), 357–367. https://doi.org/10.1046/j.1462-2920.1999.00045.x

Horn, S., Ehlers, K., Fritzsch, G., Gil-Rodríguez, M. C., Wilhelm, C., & Schnetter, R. (2007).

Synchroma grande spec. nov. (Synchromophyceae class. nov., Heterokontophyta): an amoeboid marine alga with unique plastid complexes. *Protist*, *158*(3), 277–293. https://doi.org/10.1016/j.protis.2007.02.004

Horwitz, B. Y. M. (1983). The Legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. *The Journal of Experimental Medicine*, *158*(December), 2108–2126.

Hospital, M. E. (2017). *Acanthamoeba* keratitis. Retrieved April 18, 2018, from https://www.moorfields.nhs.uk/condition/acanthamoeba-keratitis

Howland, R. B. (1928). The pH of gastric vacuoles. *Protoplasma*, *5*(1), 127–134. https://doi.org/10.1007/BF01604593

Huelsenbeck, J. P., & Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*, *17*(8), 754–755. https://doi.org/10.1093/bioinformatics/17.8.754

Hurt, M., Neelam, S., Niederkorn, J., & Alizadeh, H. (2003). Pathogenic *Acanthamoeba spp.* secrete a mannose-induced cytolytic protein that correlates with the ability to cause disease. *Infection and Immunity*, *71*(11), 6243–6255.

Huws, S. A., Morley, R. J., Jones, M. V, Brown, M. R. W., & Smith, A. W. (2008). Interactions of some common pathogenic bacteria with *Acanthamoeba polyphaga*. *FEMS Microbiology Letters*, *282*(2), 258–265. Retrieved from http://femsle.oxfordjournals.org/content/282/2/258.abstract

Huws, S. A., Smith, A. W., Enright, M. C., Wood, P. J., & Brown, M. R. W. (2006). Amoebae promote persistence of epidemic strains of MRSA. *Environmental Microbiology*, *8*(6), 1130–1133. https://doi.org/10.1111/j.1462-2920.2006.00991.x

Iovieno, A., Oechsler, R. A., Ledee, D. R., Miller, D., & Alfonso, E. C. (2010). Drug-resistant severe *Acanthamoeba* keratitis caused by rare T5 *Acanthamoeba* genotype. *Eye & Contact Lens*, *36*(3), 183–184. Retrieved from http://journals.lww.com/claojournal/Fulltext/2010/05000/Drug_Resistant_Severe_Acanthamoeba_Keratitis.11.aspx

Iqbal, J., Siddiqui, R., & Khan, N. A. (2014). *Acanthamoeba* and bacteria produce antimicrobials to target their counterpart. *Parasites & Vectors*, *7*(56). https://doi.org/10.1186/1756-3305-7-56

Irons, J. F., Dunn, M. J. G., Kefala, K., Thorn, S., Lakha, F., Caesar, D., … Gillies, M. a. (2013). The effect of a large Legionnaires' disease outbreak in Southwest Edinburgh on acute and critical care services. *The Quarterly Journal of Medicine*, *106*(12), 1087–1094. https://doi.org/10.1093/qjmed/hct167

Ishibashi, Y., Kabata, T., Hommura, S., Watanabe, R., & Ishii, K. (1992). Oral fluconazole and topical miconazole with debridement for *Acanthamoeba* keratitis. *Japanese Journal of Clinical Ophthalmology*, *46*(7), 1081–1086. https://doi.org/10.1016/S0002-9394(14)75974-4

Iwunze, M. O. (2010). Aqueous photophysical parameters of Congo Red. *Spectroscopy Letters*, *43*(1), 16–21. https://doi.org/10.1080/00387010903278226

Jackson, T. N., Tuxen, J., Heinze, J. B., & Weiner, J. M. (1986). Successful medical treatment of a corneal ulcer due to *Acanthamoeba polyphaga*. *Australian and New Zealand Journal of Ophthalmology*, *14*(2), 139–142. https://doi.org/10.1111/j.1442-9071.1986.tb00025.x

Jacobs, T., & Leippe, M. (1995). Purification and molecular cloning of a major antibacterial protein of the protozoan parasite *Entamoeba histolytica* with lysozyme-like properties. *European Journal of Biochemistry*, *231*(3), 831–838. https://doi.org/10.1111/j.1432-1033.1995.0831d.x

Jager, B. V., & Stamm, W. P. (1972). Brain abscesses caused by free-living amoeba probably of the genus *Hartmannella* in a patient with Hodgkin's disease. *The Lancet*, *300*(7791), 1343–1345. https://doi.org/10.1016/S0140-6736(72)92781-X

Jeong, H. J., Jang, E. S., Han, B. I., Lee, K. H., Ock, M. S., Kong, H. H., … Yu, H. S. (2007). *Acanthamoeba*: could it be an environmental host of *Shigella*? *Experimental Parasitology*, *115*(2), 181–186. https://doi.org/10.1016/j.exppara.2006.08.002

Jha, B. K., Seo, I., Kong, H.-H., Suh, S.-I., Suh, M.-H., & Baek, W.-K. (2015). Tigecycline inhibits

proliferation of Acanthamoeba castellanii</i>. *Parasitology Research*, *114*(3), 1189–1195. https://doi.org/10.1007/s00436-014-4302-1

Jollès, P., & Jollès, J. (1984). What's new in lysozyme research? - Always a model system, today as yesterday. *Molecular and Cellular Biochemistry*, *63*(2), 165–189. https://doi.org/10.1007/BF00285225

Jonckheere, J. F. de. (1983). Isoenzyme and total protein analysis by agarose isoelectric focusing, and taxonomy of the genus *Acanthamoeba*. *The Journal of Protozoology*, *30*(4), 701–706. https://doi.org/10.1111/j.1550-7408.1983.tb05346.x

Jones, B. R., Visvesvara, G. S., & Robinson, N. M. (1975). Acanthamoeba polyphaga keratitis and Acanthamoeba uveitis associated with fatal meningoencephalitis. *Transactions of the Ophthalmological Societies of the United Kingdom*, *95*(March), 221–232.

Kanehisa, M., Furumichi, M., Tanabe, M., Sato, Y., & Morishima, K. (2017). KEGG: new perspectives on genomes, pathways, diseases and drugs. *Nucleic Acids Research*, *45*(Database issue), D353–D361. https://doi.org/10.1093/nar/gkw1092

Kanehisa, M., & Goto, S. (2000). KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Research*, *28*(1), 27–30. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC102409/

Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M., & Tanabe, M. (2016). KEGG as a reference resource for gene and protein annotation. *Nucleic Acids Research*, *44*(Database issue), D457–D462. https://doi.org/10.1093/nar/gkv1070

Kasinathan, N., Volety, S. M., & Josyula, V. R. (2016). Chondroitinase: A promising therapeutic enzyme. *Critical Reviews in Microbiology*, *42*(3), 474–484. https://doi.org/10.3109/1040841X.2014.959893

Katoh, K., & Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Molecular Biology and Evolution*, *30*(4), 772–780. https://doi.org/10.1093/molbev/mst010

Kawabata, S. I., Tokunaga, F., Kugi, Y., Motoyama, S., Miura, Y., Hirata, M., & Iwanaga, S. (1996). Limulus factor D, a 43-kDa protein isolated from horseshoe crab hemocytes, is a serine protease homologue with antimicrobial activity. *FEBS Letters*, *398*(2–3), 146–150. https://doi.org/10.1016/S0014-5793(96)01224-0

Kelley, L. A., Mezulis, S., Yates, C. M., Wass, M. N., & Sternberg, M. J. E. (2015). The Phyre2 web portal for protein modeling, prediction and analysis. *Nature Protocols*, *10*, 845. Retrieved from http://dx.doi.org/10.1038/nprot.2015.053

Kersey, P. J., Allen, J. E., Allot, A., Barba, M., Boddu, S., Bolt, B. J., … Yates, A. (2017). Ensembl Genomes 2018: an integrated omics infrastructure for non-vertebrate species. *Nucleic Acids Research*, (November 2017), 1–7. https://doi.org/10.1093/nar/gkx1011

Key, J. E. (2007). Development of contact lenses and their worldwide use. *Eye Contact Lens*, *33*(6 Pt 2), 343. https://doi.org/10.1097/ICL.0b013e318157c230

Khan, N. A. (2001). Pathogenicity, morphology, and differentiation of *Acanthamoeba*. *Current Microbiology*, *43*(6), 391–395. https://doi.org/10.1007/s002840010325

Khan, N. A. (2006). *Acanthamoeba*: biology and increasing importance in human health. *FEMS Microbiology Reviews*, *30*(4), 564–595. https://doi.org/10.1111/j.1574-6976.2006.00023.x

Khan, N. A., Jarroll, E. L., & Paget, T. A. (2001). *Acanthamoeba* can be differentiated by the polymerase chain reaction and simple plating assays. *Current Microbiology*, *43*(3), 204–208. https://doi.org/10.1007/s002840010288

Khan, N. A., Jarroll, E. L., Panjwani, N., Cao, Z., & Paget, T. A. (2000). Proteases as markers for differentiation of pathogenic and nonpathogenic species of *Acanthamoeba*. *Journal of Clinical Microbiology*, *38*(8), 2858–2861.

Khan, N. A., & Siddiqui, R. (2014). Predator vs aliens: bacteria interactions with *Acanthamoeba*. *Parasitology*, *141*(7), 869–874. https://doi.org/10.1017/S003118201300231X

Khan, N. A., & Tareen, N. K. (2003). Genotypic, phenotypic, biochemical, physiological and

pathogenicity-based categorisation of *Acanthamoeba* strains. *Folia Parasitologica*, *50*(2), 97–104.

Khan, Y. A., Kashiwabuchi, R. T., Martins, S. A., Castro-Combs, J. M., Kalyani, S., Stanley, P., … Behrens, A. (2011). Riboflavin and ultraviolet light a therapy as an adjuvant treatment for medically refractive *Acanthamoeba* keratitis: report of 3 cases. *Ophthalmology*, *118*(2), 324–331. https://doi.org/10.1016/j.ophtha.2010.06.041

Kilvington, S. (1989). Moist-heat disinfection of pathogenic *Acanthamoeba* cysts. *Letters in Applied Microbiology*, *9*, 187–189.

Kilvington, S., Gray, T., Dart, J., Morlet, N., Beeching, J. R., Frazer, D. G., & Matheson, M. (2004). *Acanthamoeba* keratitis: The role of domestic tap water contamination in the United Kingdom. *Investigative Ophthalmology and Visual Science*, *45*(1), 165–169. https://doi.org/10.1167/iovs.03-0559

Kilvington, S., Heaselgrave, W., Lally, J. M., Ambrus, K., & Powell, H. (2008). Encystment of *Acanthamoeba* during incubation in multipurpose contact lens disinfectant solutions and experimental formulations. *Eye & Contact Lens*, *34*(3), 133–139. https://doi.org/10.1097/ICL.0b013e3181772c95

Kilvington, S., & Price, J. (1990). Survival of *Legionella pneumophila* within cysts of *Acanthamoeba polyphaga* following chlorine exposure. *Journal of Applied Bacteriology*, *68*(5), 519–525. https://doi.org/10.1111/j.1365-2672.1990.tb02904.x

Kim, H. K., Ha, Y. R., Yu, H. S., Kong, H. H., & Chung, D. Il. (2003). Purification and characterization of a 33 kDa serine protease from </i>Acanthamoeba lugdunensis</i> KA/E2 isolated from a Korean keratitis patient. *The Korean Journal of Parasitology*, *41*(4), 189–196. https://doi.org/10.3347/kjp.2003.41.4.189

Kim, W. T., Kong, H. H., Ha, Y. R., Hong, Y. C., Jeong, H. J., Yu, H. S., & Chung, D. Il. (2006). Comparison of specific activity and cytopathic effects of purified 33 kDa serine proteinase from *Acanthamoeba* strains with different degree of virulence. *The Korean Journal of Parasitology*, *44*(4), 321–330. https://doi.org/10.3347/kjp.2006.44.4.321

Kitching, J. A. (1938). Contractile Vacuoles. *Biological Reviews*, *13*(4), 403–444.

Kliescikova, J., Kulda, J., & Nohynkova, E. (2011a). Propylene glycol and contact-lens solutions containing this diol induce pseudocyst formation in acanthamoebae. *Experimental Parasitology*, *127*(1), 326–328. https://doi.org/10.1016/j.exppara.2010.08.014

Kliescikova, J., Kulda, J., & Nohynkova, E. (2011b). Stress-induced pseudocyst formation - A newly identified mechanism of protection against organic solvents in acanthamoebae of the T4 genotype. *Protist*, *162*(1), 58–69. https://doi.org/10.1016/j.protis.2010.03.006

Köhsler, M., Leitsch, D., Fürnkranz, U., Duchêne, M., Aspöck, H., & Walochnik, J. (2008). *Acanthamoeba* strains lose their abilities to encyst synchronously upon prolonged axenic culture. *Parasitology Research*, *102*(5), 1069–1072. https://doi.org/10.1007/s00436-008-0885-8

Kong, H.-H., Park, J.-H., & Chung, D.-I. (1995). Interstrain polymorphism of isoenzyme profiles and mithocondrial DNA fingerprints among seven strains assigned to *Acanthamoeba polyphaga*. *The Korean Journal of Parasitology*, *33*(4), 331–340.

Kong, H., & Chung, D. (1996). PCR and RFLP variation of conserved region of small ribosomal DNA among *Acanthamoeba* isolates assigned to either *A. castellanii* or *A. polyphaga*. *The Korean Journal of Parasitology*, *34*(2), 127–134.

Kong, H. H., Kim, T. H., & Chung, D. I. (2000). Purification and characterization of a secretory serine proteinase of *Acanthamoeba healyi* isolated from GAE. *The Journal of Parasitology*, *86*(1), 12–17.

Korn, E. D. (1974). The isolation of the amoeba plasma membrane and the use of latex beads for the isolation of phagocytic vacuole (phagosome) membranes from amoebae including the culture techniques for amoebae. *Methods in Enzymology*, *31*(C), 686–698. https://doi.org/10.1016/0076-6879(74)31074-9

Korn, E. D., & Olivecrona, T. (1971). Composition of an amoeba plasma membrane. *Biochemical*

and Biophysical Research Communications, *45*(1), 90–97.

Kosakyan, A., Heger, T. J., Leander, B. S., Todorov, M., Mitchell, E. A. D., & Lara, E. (2012). COI barcoding of nebelid testate amoebae (Amoebozoa: Arcellinida): extensive cryptic diversity and redefinition of the hyalospheniidae schultze. *Protist*, *163*(3), 415–434. https://doi.org/10.1016/j.protis.2011.10.003

Kosrirukvongs, P., Wanachiwanawin, D., & Visvesvara, G. S. (1999). Treatment of *Acanthamoeba* keratitis with chlorhexidine. *Ophthalmology*, *106*(4), 798–802. https://doi.org/http://dx.doi.org/10.1016/S0161-6420(99)90169-0

Kulkarni, M. M., McMaster, W. R., Kamysz, E., Kamysz, W., Engman, D. M., & McGwire, B. S. (2006). The major surface-metalloprotease of the parasitic protozoan, *Leishmania*, protects against antimicrobial peptide-induced apoptotic killing. *Molecular Microbiology*, *62*(5), 1484–1497. https://doi.org/10.1111/j.1365-2958.2006.05459.x

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for bigger datasets. *Molecular Biology and Evolution*, *33*(7), 1870–1874. https://doi.org/10.1093/molbev/msw054

Kuroki, T., Sata, S., Yamai, S., Yagita, K., Katsube, Y., & Endo, T. (1998). [Occurrence of free-living amoebae and *Legionella* in whirlpool bathes]. *Kansenshogaku zasshi*, *72*(10), 1056–1063.

Kusrini, E., Hashim, F., Azmi, W. N. N. W. N., Amin, N. M., & Estuningtyas, A. (2016). A novel antiamoebic agent against *Acanthamoeba sp.* - A causative agent for eye keratitis infection. *Spectrochimica Acta - Part A: Molecular and Biomolecular Spectroscopy*, *153*(3), 714–721. https://doi.org/10.1016/j.saa.2015.09.021

La Scola, B., Audic, S., Roberts, C., Jungang, L., Lamballerie, X. de, Drancourt, M., … Raoult, D. (2003). A giant virus in amoebae. *Science*, *299*(5615), 2033–2033. https://doi.org/10.1126/science.1081867

La Scola, B., & Raoult, D. (2001). Survival of *Coxiella burnetii* within fee-living amoeba *Acanthamoeba castellanii*. *Clinical Microbiology and Infection*, *7*(2), 75–79.

Larkin, D. F., Berry, M., & Easty, D. L. (1991). In vitro corneal pathogenicity of *Acanthamoeba*. *Eye*, *5*(April), 560–568. https://doi.org/10.1038/eye.1991.98

Larkin, D. F. P., Kilvington, S., & Dart, J. K. G. (1992). Treatment of Acanthamoeba keratitis with polyhexamethylene biguanide. *Ophthalmology*, *99*(2), 185–191. https://doi.org/http://dx.doi.org/10.1016/S0161-6420(92)31994-3

Laschtschenko, P. (1909). Uber die keimtötende und entwicklungshemmende Wirkung von Hühnereiweiß. *Zeitschrift Für Hygiene Und Infektionskrankheiten*, *64*(1), 419–427.

Lasjerdi, Z., Niyyati, M., Haghighi, A., Zaeri, F., & Mojarad, E. N. (2011). First report of Vannellidae amoebae (*Vannella spp.*) isolated from biofilm source. *Iranian Journal of Parasitology*, *6*(4), 84–89.

Laskowski-Arce, M. A., & Orth, K. (2008). <i>Acanthamoeba castellanii<i> promotes the survival of *Vibrio parahaemolyticus*. *Applied and Environmental Microbiology*, *74*(23), 7183–7188. https://doi.org/10.1128/AEM.01332-08

Lasman, M. (1975). Glucosidase activity in *Acanthamoeba (Mayorella) palestiniensis*. The effect of glucose and natural glucosides on alpha and beta glucosidases. *The Journal of Protozoology*, *22*(3), 435–437. https://doi.org/10.1111/j.1550-7408.1975.tb05201.x

Le Dantec, F. A. (1891). Recherches sur la digestion intracellulaire chez les protozoaires. impr. L. Danel.

Ledee, D. R., Hay, J., Byers, T. J., Seal, D. V., & Kirkness, C. M. (1996). *Acanthamoeba griffini*: molecular characterization of a new corneal pathogen. *Investigative Ophthalmology and Visual Science*, *37*(4), 544–550.

Lee, A. (2005). VirtualDub video processing software. Retrieved from citeulike-article-id:5883847

Lee, J. Y., Song, S. M., Moon, E. K., Lee, Y. R., Jha, B. K., Sylvatrie Danne, D. B., … Hong, Y. (2013). Cysteine protease inhibitor (AcStefin) is required for complete cyst formation of

Acanthamoeba. *Eukaryotic Cell*, *12*(4), 567–574. https://doi.org/10.1128/EC.00308-12

Lee, S. E., Kim, J. M., Jeong, S. K., Jeon, J. E., Yoon, H. J., Jeong, M. K., & Lee, S. H. (2010). Protease-activated receptor-2 mediates the expression of inflammatory cytokines, antimicrobial peptides, and matrix metalloproteinases in keratinocytes in response to Propionibacterium acnes. *Archives of Dermatological Research*, *302*(10), 745–756. https://doi.org/10.1007/s00403-010-1074-z

Legendre, M., Lartigue, A., Bertaux, L., Jeudy, S., Bartoli, J., Lescot, M., … Claverie, J.-M. (2015). In-depth study of *Mollivirus sibericum*, a new 30,000-y-old giant virus infecting *Acanthamoeba*. *Proceedings of the National Academy of Sciences*, *112*(38), E5327–E5335. https://doi.org/10.1073/pnas.1510795112

Leher, H., Silvany, R., Alizadeh, H., Huang, J., & Niederkorn, J. Y. (1998). Mannose induces the release of cytopathic factors from *Acanthamoeba castellanii*. *Infection and Immunity*, *66*(1), 5–10.

Leippe, M. (1999). Antimicrobial and cytolytic polypeptides of amoeboid protozoa - effector molecules of primitive phagocytes. *Developmental and Comparative Immunology*, *23*(4–5), 267–279. https://doi.org/10.1016/S0145-305X(99)00010-5

Leippe, M. (2014). Pore-forming toxins from pathogenic amoebae. *Applied Microbiology and Biotechnology*, *98*(10), 4347–4353. https://doi.org/10.1007/s00253-014-5673-z

Leippe, M., Ebel, S., Schoenberger, O. L., Horstmann, R. D., & Müller-Eberhard, H. J. (1991). Pore-forming peptide of pathogenic *Entamoeba histolytica*. *Proceedings of the National Academy of Sciences of the United States of America*, *88*(17), 7659–7663. https://doi.org/10.1073/pnas.88.17.7659

Leitsch, D., Köhsler, M., Marchetti-Deschmann, M., Deutsch, A., Allmaier, G., Duchêne, M., & Walochnik, J. (2010). Major role for cysteine proteases during the early phase of *Acanthamoeba castellanii* encystment. *Eukaryotic Cell*, *9*(4), 611–618. https://doi.org/10.1128/EC.00300-09

Letcher, P. M., Longcore, J. E., Quandt, C. A., Leite, D. da S., James, T. Y., & Powell, M. J. (2017). Morphological, molecular, and ultrastructural characterization of *Rozella rhizoclosmatii*, a new species in Cryptomycota. *Fungal Biology*, *121*(1), 1–10. https://doi.org/10.1016/j.funbio.2016.08.008

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., … Durbin, R. (2009). The Sequence Alignment/Map format and SAMtools. *Bioinformatics*, *25*(16), 2078–2079. https://doi.org/10.1093/bioinformatics/btp352

Liao, Y., Smyth, G. K., & Shi, W. (2014). FeatureCounts: An efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*, *30*(7), 923–930. https://doi.org/10.1093/bioinformatics/btt656

Liu, H., Moon, E. K., Yu, H. S., Jeong, H. J., Hong, Y. C., Kong, H. H., & Chung, D. Il. (2005). Evaluation of taxonomic validity of four species of *Acanthamoeba: A. divionensis, A. paradivionensis, A. mauritaniensis*, and *A. rhysodes*, inferred from molecular analyses. *The Korean Journal of Parasitology*, *43*(1), 7–13. https://doi.org/200503007 [pii]

Lloyd, D. (2014). Encystment in *Acanthamoeba castellanii*: A review. *Experimental Parasitology*, *145*(S), S20–S27. https://doi.org/10.1016/j.exppara.2014.03.026

López-Escardó, D., López-García, P., Moreira, D., Ruiz-Trillo, I., & Torruella, G. (2017). *Parvularia atlantis gen. et sp. nov.*, a nucleariid filose amoeba (Holomycota, Opisthokonta). *Journal of Eukaryotic Microbiology*, *0*, 1–10. https://doi.org/10.1111/jeu.12450

Lorch, J. (1973). Some historical aspects of amoeba studies. In Kwang W. Jeon (Ed.), *The Biology of Amoeba* (1st ed.). Knoxville: Academic Press. Retrieved from https://books.google.co.uk/books?id=J0vniMFGPukC&printsec=frontcover&dq=amoeba&hl=en&sa=X&redir_esc=y#v=onepage&q=amoeba&f=false

Lorenzo-Morales, J., Coronado-Álvarez, N., Martínez-Carretero, E., Maciver, S. K., & Valladares, B. (2007). Detection of four adenovirus serotypes within water-isolated strains of *Acanthamoeba* in the Canary Islands, Spain. *American Journal of Tropical Medicine and Hygiene*, *77*(4), 753–756.

Lorenzo-Morales, J., Khan, N. A., & Walochnik, J. (2015). An update on *Acanthamoeba* keratitis: diagnosis, pathogenesis and treatment. *Parasite*, *22*(10). https://doi.org/10.1051/parasite/2015010

Lorenzo-Morales, J., Kliescikova, J., Martinez-Carretero, E., De Pablos, L. M., Profotova, B., Nohynkova, E., … Valladares, B. (2008). Glycogen phosphorylase in *Acanthamoeba spp.*: determining the role of the enzyme during the encystment process using RNA interference. *Eukaryotic Cell*, *7*(3), 509–517. https://doi.org/10.1128/EC.00316-07

Lorenzo-Morales, J., Lindo, J. F., Martinez, E., Calder, D., Figueruelo, E., Valladares, B., & Ortega-Rivas, A. (2005a). Pathogenic *Acanthamoeba* strains from water sources in Jamaica, West Indies. *Annals of Tropical Medicine & Parasitology*, *99*(8), 751–758. https://doi.org/10.1179/136485905X65215

Lorenzo-Morales, J., Martín-Navarro, C. M., López-Arencibia, A., Arnalich-Montiel, F., Piñero, J. E., & Valladares, B. (2013). Acanthamoeba keratitis: an emerging disease gathering importance worldwide? *Trends in Parasitology*, *29*(4), 181–187. https://doi.org/10.1016/j.pt.2013.01.006

Lorenzo-Morales, J., Ortega-Rivas, A., Foronda, P., Abreu-Acosta, N., Ballart, D., Martinez, E., & Valladares, B. (2005b). RNA interference (RNAi) for the silencing of extracellular serine proteases genes in Acanthamoeba: Molecular analysis and effect on pathogenecity. *Molecular and Biochemical Parasitology*, *144*, 10–15. https://doi.org/10.1016/j.molbiopara.2005.07.001

Lorenzo-Morales, J., Reyes-Batlle, M., Sifaoui, I., Arnalich-Montiel, F., López-Arencibia, A., Wagner, C., … Valladares, B. (2016). Therapeutic targets and investigated treatment strategies in *Acanthamoeba* keratitis. *Expert Opinion on Orphan Drugs*, *4*(10), 1069–1073. https://doi.org/10.1080/21678707.2016.1230060

Luo, W., Friedman, M. S., Shedden, K., Hankenson, K. D., & Woolf, P. J. (2009). GAGE: generally applicable gene set enrichment for pathway analysis. *BMC Bioinformatics*, *10*(161). https://doi.org/10.1186/1471-2105-10-161

Maciver, S. K. (2016). Asexual amoebae escape Muller's ratchet through polyploidy. *Trends in Parasitology*, *32*(11), 855–862. https://doi.org/10.1016/j.pt.2016.08.006

Maciver, S. K., Asif, M., McCalister, K., & de Obeso Fernandez del Valle, A. (2015a). Reviving the ability to encyst in a long established axenic strain of Acanthamoeba: Evidence for a common pathway for various encysting stimuli. In A. Mattana (Ed.), *XVI International Meeting on the Biology and Pathogenicity of Free-Living Amoebae (FLAM 2015)* (p. 39). Alghero.

Maciver, S. K., Asif, M., Simmen, M. W., & Lorenzo-Morales, J. (2013). A systematic analysis of *Acanthamoeba* genotype frequency correlated with source and pathogenicity: T4 is confirmed as a pathogen-rich genotype. *European Journal of Protistology*, *49*(2), 217–221. https://doi.org/10.1016/j.ejop.2012.11.004

Maciver, S. K., de Obeso Fernandez del Valle, A., & Koutsogiannis, Z. (2017). Vannella pentlandii n. sp., (Amoebozoa, Discosea, Vannellida) a small, cyst-forming soil amoeba. *Experimental Parasitology*, *183*, 109–116. https://doi.org/10.1016/j.exppara.2017.07.011

Maciver, S. K., McCalister, K., & de Obeso Fernandez del Valle, A. (2015b). The last amoeba standing. Cannibalism as a mechanism for the loss of the ability to encyst in axenic cultures of *Acanthamoeba*. In A. Mattana (Ed.), *XVI International Meeting on the Biology and Pathogenicity of Free-Living Amoebae (FLAM 2015)* (p. 40). Alghero.

Magnet, A., Peralta, R. H. S., Gomes, T. S., Izquierdo, F., Fernandez-Vadillo, C., Galvan, a L., & Pozuelo, M. J. (2015). Vectorial role of *Acanthamoeba* in Legionella propagation in water for human use. *Science of the Total Environment*, *505*, 889–895. https://doi.org/10.1016/j.scitotenv.2014.10.064

Makioka, a, Kumagai, M., Ohtomo, H., Kobayashi, S., & Takeuchi, T. (2000). *Entamoeba invadens*: protein kinase C inhibitors block the growth and encystation. *Experimental Parasitology*, *95*(4), 288–290. https://doi.org/10.1006/expr.2000.4538

Makioka, A., Kumagai, M., Ohtomo, H., Kobayashi, S., & Takeuchi, T. (2001). Effect of calcium

antagonists, calcium channel blockers and calmodulin inhibitors on the growth and encystation of *Entamoeba histolytica* and *E. invadens*. *Parasitology Research*, *87*(10), 833–837. https://doi.org/10.1007/s004360100453

Makioka, A., Kumagai, M., Ohtomo, H., Kobayashi, S., & Takeuchi, T. (2002). Effect of proteasome inhibitors on the growth, encystation, and excystation of *Entamoeba histolytica* and *Entamoeba invadens*. *Parasitology Research*, *88*(5), 454–459. https://doi.org/10.1007/s00436-002-0601-z

Malla, N., & Goyal, K. (2016). Ocular parasitic infections - An overview. In S. Rumelt (Ed.), *Advances in Common Eye Infections* (pp. 41–98). InTech. https://doi.org/10.5772/64137

Marciano-Cabral, F., & Cabral, G. (2003). *Acanthamoeba spp.* as agents of disease in humans. *Clinical Microbiology Reviews*, *16*(2), 273–307. https://doi.org/10.1128/CMR.16.2.273-307.2003

Martín-Navarro, C. M., Lorenzo-Morales, J., Machin, R. P., López-Arencibia, A., García-Castellano, J. M., De Fuentes, I., … Piñero, J. E. (2013). Inhibition of 3-hydroxy-3-methylglutaryl-coenzyme a reductase and application of statins as a novel effective therapeutic approach against *Acanthamoeba* infections. *Antimicrobial Agents and Chemotherapy*, *57*(1), 375–381. https://doi.org/10.1128/AAC.01426-12

Martinez, A. J. (1980). Is *Acanthamoeba* encephalitis an opportunistic infection? *Neurology*, *30*(6), 567. https://doi.org/10.1212/WNL.30.6.567

Martinez, A. J. (1991). Infection of the central nervous system due to *Acanthamoeba*. *Reviews of Infectious Diseases*, *13*(5), S399-402. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2047674

Martinez, M. S., Gonzalez-Mediero, G., Santiago, P., Rodriguez de Lope, A., Diz, J., Conde, C., & Visvesvara, G. S. (2000). Granulomatous amebic encephalitis in a patient with AIDS: isolation of *Acanthamoeba sp.* group II from brain tissue and successful treatment with sulfadiazine and fluconazole. *Journal of Clinical Microbiology*, *38*(10), 3892–3895.

Maschio, V. J., Chies, F., Carlesso, A. M., Carvalho, A., Rosa, S. P., Van Der Sand, S. T., & Rott, M. B. (2014). *Acanthamoeba* T4, T5 and T11 isolated from mineral water bottles in southern Brazil. *Current Microbiology*, *70*(1), 6–9. https://doi.org/10.1007/s00284-014-0676-7

Maschio, V. J., Corção, G., Bücker, F., Caumo, K., & Rott, M. B. (2015). Identification of *Paenibacillus* as a symbiont in *Acanthamoeba*. *Current Microbiology*, *71*(3), 415–420. https://doi.org/10.1007/s00284-015-0869-8

Masschalck, B., & Michiels, C. W. (2003). Antimicrobial properties of lysozyme in relation to foodborne vegetative bacteria. *Critical Reviews in Microbiology*, *29*(3), 191–214. https://doi.org/10.1080/713610448

Mathers, W., Stevens Jr., G., Rodrigues, M., Chan, C. C., Gold, J., Visvesvara, G. S., … Zimmerman, L. E. (1987). Immunopathology and electron microscopy of *Acanthamoeba* keratitis. *American Journal of Ophthalmology*, *103*(5), 626–635. https://doi.org/10.1016/S0002-9394(14)74321-1

Matin, A., & Jung, S.-Y. (2011). Phospholipase activities in clinical and environmental isolates of *Acanthamoeba*. *The Korean Journal of Parasitology*, *49*(1), 1.

Mattana, A., Serra, C., Mariotti, E., Delogu, G., Fiori, P. L., & Cappuccinelli, P. (2006). *Acanthamoeba castellanii* promotion of in vitro survival and transmission of coxsackie b3 viruses. *Eukaryotic Cell*, *5*(4), 665–671. https://doi.org/10.1128/EC.5.4.665-671.2006

Mauduit, P., Herman, G., & Rossignol, B. (1984). Protein secretion induced by isoproterenol or pentoxifylline in lacrimal gland : Ca2 + effects. *American Journal of Physiology*, *246*(1), C37–C44.

Mayes, D. F., Rogerson, A., Marchant, H., & Laybourn-Parry, J. (1997). Growth and consumption rates of bacterivorous Antarctic naked marine amoebae. *Marine Ecology Progress Series*, 101–108.

McCalister, K. (2015). *The* Acanthamoeba *cyst and pseudocyst*. University of Edinburgh.

McFadden, G. I., Gilson, P. R., Hofmann, C. J., Adcock, G. J., & Maier, U. G. (1994). Evidence

that an amoeba acquired a chloroplast by retaining part of an engulfed eukaryotic alga. *Proceedings of the National Academy of Sciences of the United States of America*, *91*(9), 3690–3694. https://doi.org/10.1073/pnas.91.9.3690

Medina, M., Collins, A. G., Taylor, J. W., Valentine, J. W., Lipps, J. H., Amaral-Zettler, L., & Sogin, M. L. (2003). Phylogeny of Opisthokonta and the evolution of multicellularity and complexity in Fungi and Metazoa. *International Journal of Astrobiology*, *2*(3), 203–211. https://doi.org/10.1017/S1473550403001551

Michalek, M., Sönnichsen, F. D., Wechselberger, R., Dingley, A. J., Hung, C.-W., Kopp, A., … Leippe, M. (2013). Structure and function of a unique pore-forming protein from a pathogenic *Acanthamoeba*. *Nature Chemical Biology*, *9*(1), 37–42. https://doi.org/10.1038/nchembio.1116

Michel, R., & Hauröder, B. (1997). Isolation of an *Acanthamoeba* strain with intracellular *Burkholderia pickettii* infection. *Zentralblatt Für Bakteriologie*, *285*(4), 541–557. https://doi.org/10.1016/S0934-8840(97)80116-8

Michel, R., Schmid, E. N., Boker, T., Hager, D. G., Muller, K. D., Hoffmann, R., & Seitz, H. M. (2000). *Vannella sp.* harboring Microsporidia-like organisms isolated from the contact lens and inflamed eye of a female keratitis patient. *Parasitology Research*, *86*(6), 514–520. Retrieved from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10894481

Mitra, M. M., Alizadeh, H., Gerard, R. D., & Niederkorn, J. Y. (1995). Characterization of a plasminogen activator produced by *Acanthamoeba castellanii*. *Molecular and Biochemical Parasitology*, *73*(94), 157–164. https://doi.org/10.1016/0166-6851(94)00109-Z

Mitro, K., Bhagavathiammai, A., Zhou, O. M., Bobbett, G., McKerrow, J. H., Chokshi, R., … James, E. R. (1994). Partial characterization of the proteolytic secretions of *Acanthamoeba polyphaga*. *Experimental Parasitology*, *78*, 377–385. https://doi.org/10.1006/expr.1994.1041

Moliner, C., Raoult, D., & Fournier, P. E. (2009). Evidence of horizontal gene transfer between amoeba and bacteria. *Clinical Microbiology and Infection*, *15*(SUPPL. 2), 178–180. https://doi.org/10.1111/j.1469-0691.2008.02216.x

Moon, E.-K., Hong, Y., Chung, D.-I., Goo, Y.-K., & Kong, H.-H. (2015). Potential value of cellulose synthesis inhibitors combined with PHMB in the treatment of *Acanthamoeba* keratitis. *Cornea*, *34*(12), 1593–1598. Retrieved from http://journals.lww.com/corneajrnl/Fulltext/2015/12000/Potential_Value_of_Cellulose_Synthesis_Inhibitors.14.aspx

Moon, E.-K., Xuan, Y.-H., Chung, D.-I., Hong, Y., & Kong, H.-H. (2011). Microarray analysis of differentially expressed genes between cysts and trophozoites of </i>Acanthamoeba castellanii</i>. *The Korean Journal of Parasitology*, *49*(4), 341–347. https://doi.org/10.3347/kjp.2011.49.4.341

Moon, E. K., Chung, D. I., Hong, Y. C., Ahn, T. I., & Kong, H. H. (2008a). Acanthamoeba castellanii: gene profile of encystation by ESTs analysis and KOG assignment. *Experimental Parasitology*, *119*(1), 111–116. https://doi.org/10.1016/j.exppara.2008.01.001

Moon, E. K., Chung, D. Il, Hong, Y. C., & Kong, H. H. (2007). Differentially expressed genes of *Acanthamoeba castellanii* during encystation. *The Korean Journal of Parasitology*, *45*(4), 283–285. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3279671&tool=pmcentrez&rendertype=abstract

Moon, E. K., Chung, D. Il, Hong, Y. C., & Kong, H. H. (2008b). Characterization of a serine proteinase mediating encystation of *Acanthamoeba*. *Eukaryotic Cell*, *7*(9), 1513–1517. https://doi.org/10.1128/EC.00068-08

Moon, E. K., Chung, D. Il, Hong, Y. C., & Kong, H. H. (2009). Autophagy protein 8 mediating autophagosome in encysting *Acanthamoeba*. *Molecular and Biochemical Parasitology*, *168*(1), 43–48. https://doi.org/10.1016/j.molbiopara.2009.06.005

Moon, E. K., Hong, Y., Chung, D. Il, Goo, Y. K., & Kong, H. H. (2016). Identification of protein

arginine methyltransferase 5 as a regulator for encystation of *Acanthamoeba*. *Korean Journal of Parasitology*, *54*(2), 133–138. https://doi.org/10.3347/kjp.2016.54.2.133

Moon, E. K., Hong, Y., Chung, D. Il, & Kong, H. H. (2012). Cysteine protease involving in autophagosomal degradation of mitochondria during encystation of *Acanthamoeba*. *Molecular and Biochemical Parasitology*, *185*(2), 121–126. https://doi.org/10.1016/j.molbiopara.2012.07.008

Moon, E. K., & Kong, H. H. (2012). Short-cut pathway to synthesize cellulose of encysting *Acanthamoeba*. *Korean Journal of Parasitology*, *50*(4), 361–364. https://doi.org/10.3347/kjp.2012.50.4.361

Moore, M. B., McCulley, J. P., Luckenbach, M., Gelender, H., Newton, C., McDonald, M. B., & Visvesvara, G. S. (1985). *Acanthamoeba* keratitis associated with soft contact lenses. *American Journal of Ophthalmology*, *100*(3), 396–403. https://doi.org/10.1016/0002-9394(85)90500-8

Moran, D. M., Anderson, O. R., Dennett, M. R., Caron, D. A., & Gast, R. J. (2007). A description of seven Antarctic marine Gymnamoebae including a new species and a new genus: Platyamoeba contorta n. sp. and Vermistella antarctica n. gen. n. sp</i>. *Journal of Eukaryotic Microbiology*, *54*, 169–183.

Moreno-Mesonero, L., Moreno, Y., Alonso, J. L., & Ferrús, M. A. (2016). DVC-FISH and PMA-qPCR techniques to assess the survival of *Helicobacter pylori* inside *Acanthamoeba castellanii*. *Research in Microbiology*, *167*(1), 29–34. https://doi.org/10.1016/j.resmic.2015.08.002

Mortazavi, P. N., Keisary, E., Loh, L. N., Jung, S.-Y., & Khan, N. A. (2011). Possible roles of phospholipase A 2 in the biological activities of *Acanthamoeba castellanii* (T4 genotype). *Protist*, *162*(1), 168–176.

Mulec, J., Dietersdorfer, E., Üstüntürk-Onan, M., & Walochnik, J. (1983). *Acanthamoeba* and other free-living amoebae in bat guano, an extreme habitat. *Parasitology Research*, *115*(4), 1375–1383. https://doi.org/10.1007/s00436-015-4871-7

Muller, M. (1969). Lysosomal hydrolases in *Acanthamoeba sp. Journal of Eukaryotic Microbiology*, *16*(3), 428–431.

Na, B. K., Kim, J. C., & Song, C. Y. (2001). Characterization and pathogenetic role of proteinase from *Acanthamoeba castellanii*. *Microbial Pathogenesis*, *30*, 39–48. https://doi.org/10.1006/mpat.2000.0403

Nagington, J., & Richards, J. E. (1976). Chemotherapeutic compounds and *Acanthamoebae* from eye infections. *Journal of Clinical Pathology*, *29*(7), 648–651. https://doi.org/10.1136/jcp.29.7.648

Nagington, J., Watson, P. G., Playfair, T. J., Mcgill, J., Jones, B., & Steele, A. D. M. (1974). Amoebic infection of the eye. *The Lancet*, *304*(7896), 1537–1540. https://doi.org/10.1016/S0140-6736(74)90285-2

Nassonova, E., Smirnov, A., Fahrni, J., & Pawlowski, J. (2010). Barcoding amoebae: comparison of SSU, ITS and COI genes as tools for molecular identification of naked lobose amoebae. *Protist*, *161*(1), 102–115. https://doi.org/10.1016/j.protis.2009.07.003

NCBI. (2015). *Acanthamoeba*. *National Center for Biotechnology Information*. Retrieved from https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Info&id=5754&lvl=3&lin=f&keep=1&srchmode=1&unlock

Neelam, S., & Niederkorn, J. Y. (2017). Pathobiology and immunobiology of *Acanthamoeba* keratitis: insights from animal models. *Yale Journal of Biology and Medicine*, *90*, 261–268.

Neff, R. J., Benton, W. F., & Neff, R. H. (1964a). Composition of mature cyst wall of soil ameba *Acanthamoeba sp.* In *Journal of Cell Biology* (Vol. 23, p. A66).

Neff, R. J., Ray, S. A., Benton, W. F., & Wilborn, M. (1964b). Chapter 4 Induction of synchronous encystment (differentiation) in *Acanthamoeba sp.* In *Methods in Cell Biology* (pp. 55–83). https://doi.org/10.1016/S0091-679X(08)62086-5

Neilson, J. B., Ivey, M. H., & Bulmer, G. S. (1978). Cryptococcus neoformans: pseudohyphal

forms surviving culture with *Acanthamoeba polyphaga*. *Infection and Immunity*, *20*(1), 262–266.

Niyyati, M., Lorenzo-Morales, J., Rezaie, S., Rahimi, F., Martín-Navarro, C. M., Mohebali, M., … Rezaeian, M. (2010). First report of a mixed infection due to *Acanthamoeba* genotype T3 and *Vahlkampfia* in a cosmetic soft contact lens wearer in Iran. *Experimental Parasitology*, *126*(1), 89–90. https://doi.org/10.1016/j.exppara.2009.10.009

Nowack, E. C. M., Melkonian, M., & Glöckner, G. (2008). Chromatophore genome sequence of *Paulinella* sheds light on acquisition of photosynthesis by eukaryotes. *Current Biology*, *18*(6), 410–418. https://doi.org/10.1016/j.cub.2008.02.051

Nuprasert, W., Putaporntip, C., Pariyakanok, L., & Jongwutiwes, S. (2010). Identification of a novel T17 genotype of *Acanthamoeba* from environmental isolates and T10 genotype causing keratitis in Thailand. *Journal of Clinical Microbiology*, *48*(12), 4636–4640. https://doi.org/10.1128/JCM.01090-10

Omaña-Molina, M., González-Robles, A., Iliana Salazar-Villatoro, L., Lorenzo-Morales, J., Cristóbal-Ramos, A. R., Hernández-Ramírez, V. I., … Martínez-Palomo, A. (2013). Reevaluating the role of *Acanthamoeba* proteases in tissue invasion: observation of cytopathogenic mechanisms on MDCK cell monolayers and hamster corneal cells. *BioMed Research International*, *2013*. https://doi.org/10.1155/2013/461329

Optometry-Today. (2016, September 4). ACLM's 2016 Year Book stats: four million and counting. Retrieved from http://go.galegroup.com/ps/i.do?p=AONE&sw=w&u=ed_itw&v=2.1&it=r&id=GALE%7CA462982021&asid=7e184b103a6c94960a41d6f784517f51

Page, F. C. (1967). Re-definition of the genus *Acanthamoeba* with descriptions of three species. *The Journal of Protozoology*, *14*(4), 709–724. https://doi.org/10.1111/j.1550-7408.1967.tb02066.x

Page, F. C. (1968). Generic criteria for *Flabellula, Rugipes* and *Hyalodiscus*, with description of species. *Journal of Protozoology*, *15*(1), 9–26.

Page, F. C. (1974). Some marine *Platyamoeba* of East Anglia. *Journal of the Marine Biological Association of the United Kingdom*, *54*(3), 651–664.

Page, F. C. (1980). A key to marine species of *Vannella* (Sarcodina: Gymnamoebia), with descriptions of new species. *Journal of the Marine Biological Association of the United Kingdom*, *60*(4), 929–946.

Page, F. C. (1983). *Marine gymnamoeba*. Lavenham, Suffolk: Intitute of Terrestrial Ecology.

Page, F. C. (1987). The classification of 'naked' amoebae (Phylum Rhizopoda). *Archiv Fur Protistenkunde*, *133*(3–4), 199–217. https://doi.org/10.1016/S0003-9365(87)80053-2

Page, F. C., & Blanton, R. L. (1985). The Heterolobosea (Sarcodina: Rhizopoda), a new class uniting the Schizopyrenida and the Acrasidae (Acrasida). *Protistologica*, *21*(1), 121–132.

Pánek, T., & Čepicka, I. (2012). Diversity of Heterolobosea. In InTech (Ed.), *Genetic Diversity in Microorganisms* (pp. 3–26). https://doi.org/10.5772/2641

Pánek, T., Simpson, A. G. B., Brown, M. W., & Dexter Dyer, B. (2017). Heterolobosea. In J. M. Archibald, A. G. B. Simpson, C. H. Slamovits, L. Margulis, M. Melkonian, D. J. Chapman, & J. O. Corliss (Eds.), *Handbook of the Protists* (pp. 1–42). Cham: Springer International Publishing. https://doi.org/10.1007/978-3-319-32669-6_10-1

Panjwani, N. (2010). Pathogenesis of *Acanthamoeba keratitis*. *The Ocular Surface*, *8*(2), 70–79. https://doi.org/10.1016/S1542-0124(12)70071-X

Park, D. H., Palay, D. A., Daya, S. M., Stulting, R. D., Krachmer, J. H., & Holland, E. J. (1997). The role of topical corticosteroids in the management of *Acanthamoeba* keratitis. *Cornea*, *16*(3), 277–283. Retrieved from http://journals.lww.com/corneajrnl/Fulltext/1997/05000/The_Role_of_Topical_Corticosteroids_in_the.4.aspx

Paszko-Kolva, C., Yamamoto, H., Shahamat, M., Sawyer, T. K., Morris, G., & Colwell, R. R. (1991). Isolation of amoebae and *Pseudomonas* and *Legionella spp.* from eyewash

stations. *Applied and Environmental Microbiology*, *57*(1), 163–167.

Patil, V., Bråte, J., Shalchian-Tabrizi, K., & Jakobsen, K. S. (2009). Revisiting the phylogenetic position of *Synchroma grande*. *Journal of Eukaryotic Microbiology*, *56*(4), 394–396. https://doi.org/10.1111/j.1550-7408.2009.00389.x

Phillipe, N., Legendre, M., Doutre, G., Poirot, O., Lescot, M., Arslan, D., … Claverie, J. (2013). Pandoraviruses: amoeba viruses with genomes up to 2.5 Mb reaching that of parasitic eukaryotes. *Science*, *341*(July), 281–286. https://doi.org/10.1126/science.1239181

Piña-Vázquez, C., Reyes-López, M., Ortíz-Estrada, G., De La Garza, M., & Serrano-Luna, J. (2012). Host-parasite interaction: parasite-derived and -induced proteases that degrade human extracellular matrix. *Journal of Parasitology Research*, *2012*, 1–24. https://doi.org/10.1155/2012/748206

Pollard, T. D., Magnus, K. A., Doberstein, S., Goldschmidt-Clermont, P., Kaiser, D. A., Machesky, L., … Wachsstock, D. (1989). Structure-function studies of the actin filament system of Acanthamoeba. In *Cytoskeletal and Extracellular Proteins* (pp. 271–279). Springer.

Potter, J. L., & Weisman, R. A. (1971). Differentiation in *Acanthamoeba*: beta-glucan synthesis during encystment. *Biochimica et Biophysica Acta*, *237*, 65–74.

Potter, J. L., & Weisman, R. A. (1972). Correlation of cellulose synthesis in vivo and in vitro during the encystment of *Acanthamoeba*. *Developmental Biology*, *28*(3), 472–479. https://doi.org/10.1016/0012-1606(72)90030-9

Preston, T. M., & King, C. A. (1984). Amoeboid locomotion of *Acanthamoeba castellanii* with special reference to cell substratum interactions. *Journal of General Microbiology*, *130*(23), 17–2323. https://doi.org/10.1099/00221287-130-9-2317

Preston, T. M., Richards, H., & Wotton, R. S. (2001). Locomotion and feeding of *Acanthamoeba* at the water-air interface of ponds. *FEMS Microbiology Letters*, *194*(2), 143–147. https://doi.org/10.1016/S0378-1097(00)00517-6

Pussard, M., & Pons, R. (1977). Morphology of cystic wall and taxonomy of genus *Acanthamoeba* (Protozoa, Amoebida). *Protistologica*, *13*(4), 557–598. Retrieved from http://apps.webofknowledge.com/full_record.do?product=UA&search_mode=GeneralSearch&qid=1&SID=S2jKFgWJrPtokT7atuH&page=1&doc=2

Qureshi, M. N., Perez, a a, Madayag, R. M., & Bottone, E. J. (1993). Inhibition of *Acanthamoeba* species by *Pseudomonas aeruginosa*: rationale for their selective exclusion in corneal ulcers and contact lens care systems. *Journal of Clinical Microbiology*, *31*(7), 1908–1910.

Qvarnstrom, Y., Nerad, T. A., & Visvesvara, G. S. (2013). Characterization of a new pathogenic *Acanthamoeba* species, *A. byersi n. sp.*, isolated from a human with fatal amoebic encephalitis. *Journal of Eukaryotic Microbiology*, *60*(6), 626–633. https://doi.org/10.1001/jamainternmed.2014.5466.Association

Radford, C. F., Minassian, D. C., & Dart, J. K. G. (2002). *Acanthamoeba* keratitis in England and Wales: incidence, outcome, and risk factors. *British Journal of Ophthalmology*, *86*(5), 536–542. https://doi.org/10.1136/bjo.86.5.536

Rama, P., Matuska, S., Viganò, M., Spinelli, A., Paganoni, G., & Brancato, R. (2003). Bilateral *Acanthamoeba* keratitis with late recurrence of the infection in a corneal graft: a case report. *European Journal of Ophthalmology*, *13*(3), 311–314.

Ramírez-Rico, G., Martínez-Castillo, M., De La Garza, M., Shibayama, M., & Serrano-Luna, J. (2015). *Acanthamoeba castellanii* proteases are capable of degrading iron-binding proteins as a possible mechanism of pathogenicity. *Journal of Eukaryotic Microbiology*, *62*(5), 614–622. https://doi.org/10.1111/jeu.12215

Ramirez, E., Robles, E., & Martinez, B. (2010). Free-living amoebae isolated from water-hyacinth root (*Eichhornia crassipes*). *Experimental Parasitology*, *126*(1), 42–44. https://doi.org/10.1016/j.exppara.2010.01.020

Ramirez, E., Robles, E., Martinez, B., Ayala, R., Sainz, G., Martinez, M. E., & Gonzalez, M. E. (2014). Distribution of free-living amoebae in a treatment system of textile industrial wastewater. *Experimental Parasitology*, *145*(S), S34–S38.

https://doi.org/10.1016/j.exppara.2014.07.006

Resnikoff, S., Paniagua-Crespo, E., Mjikam, J. M., & Simitzis-Le Flohic, A. M. (1991). First cases of keratitis caused by free-living amoebas of the genus *Acanthamoeba* diagnosed in Mali. *Bulletin De La Societe De Pathologie Exotique*, *84*(5 Pt 5), 1016–1020. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1819387

Reyes-Batlle, M., Todd, C. D., Martín-Navarro, C. M., López-Arencibia, A., Cabello-Vilchez, A. M., González, A. C., … Lorenzo-Morales, J. (2014). Isolation and characterization of *Acanthamoeba* strains from soil samples in Gran Canaria, Canary Islands, Spain. *Parasitology Research*, *113*(4), 1383–1388. https://doi.org/10.1007/s00436-014-3778-z

Rezaeian, M., Niyyati, M., & Motevalli-Haghi, A. (2008). Isolation of *Acanthamoeba spp.* from different environmental sources. *Iranian Journal of Parasitology*, *3*(1), 44–47. https://doi.org/10.1099/jmm.0.065466-0

Rimm, D. L., Pollard, T. D., & Hieter, P. (1988). Resolution of *Acanthamoeba castellanii* chromosomes by pulsed field gel electrophoresis and construction of the initial linkage map. *Chromosoma*, *97*(3), 219–223. https://doi.org/10.1007/BF00292964

Ritchie, M. E., Phipson, B., Wu, D., Hu, Y., Law, C. W., Shi, W., & Smyth, G. K. (2015). limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Research*, *43*(7), e47. https://doi.org/10.1093/nar/gkv007

Rivera, F., Galvan, M., Robles, E., Leal, P., Gonzalez, L., & Lacy, A. M. (1981). Bottled mineral waters polluted by protozoa in Mexico. *The Journal of Protozoology*, *28*(1), 54–56. https://doi.org/10.1111/j.1550-7408.1981.tb02803.x

Rivera, M. A., & Padhya, T. A. (2002). *Acanthamoeba*: a rare primary cause of rhinosinusitis. *The Laryngoscope*, *112*(7), 1201–1203. https://doi.org/10.1097/00005537-200207000-00011

Robinson, J. T., Thorvaldsdottir, H., Winckler, W., Guttman, M., Lander, E. S., Getz, G., & Mesirov, J. P. (2013). Integrative Genomics Viewer. *Nature Biotechnology*, *29*(1), 24–26. https://doi.org/10.1038/nbt0111-24

Robinson, M. D., McCarthy, D. J., & Smyth, G. K. (2009). edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, *26*(1), 139–140. https://doi.org/10.1093/bioinformatics/btp616

Robinson, M. D., & Oshlack, A. (2010). A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biology*, *11*(3), R25. https://doi.org/10.1186/gb-2010-11-3-r25

Roger, A. J., Smith, M. W., Doolittle, R. F., & Doolittle, W. F. (1996). Evidence for the heterolobosea from phylogenetic analysis of genes encoding glyceraldehyde-3-phosphate dehydrogenase. *Journal of Eukaryotic Microbiology*, *43*(6), 475–485.

Ronquist, F., & Huelsenbeck, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, *19*(12), 1572–1574. https://doi.org/10.1093/bioinformatics/btg180

Rosano, G. L., & Ceccarelli, E. A. (2014). Recombinant protein expression in *Escherichia coli*: Advances and challenges. *Frontiers in Microbiology*, *5*, 1–17. https://doi.org/10.3389/fmicb.2014.00172

Rosenberg, K., Bertaux, J., Krome, K., Hartmann, A., Scheu, S., & Bonkowski, M. (2009). Soil amoebae rapidly change bacterial community composition in the rhizosphere of *Arabidopsis thaliana*. *The ISME Journal*, *3*(6), 675–684. https://doi.org/10.1038/ismej.2009.11

Rosenthal, S., Reed, E. J., & Weisman, R. A. (1969). Effect of lytic enzymes of *Acanthamoeba castellanii* on bacterial cell walls. *Journal of Bacteriology*, *98*(1), 182–189.

Rowbotham, T. J. (1980). Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. *Journal of Clinical Pathology*, *33*(12), 1179–1183. https://doi.org/10.1136/jcp.33.12.1179

Rubin, R. W., Hill, M. C., Hepworth, P., & Boehmer, J. (1976). Isolation and electrophoretic analysis of nucleoli, phenol-soluble nuclear proteins and outer cyst walls from *Acanthamoeba castellanii* during encystation initiation. *The Journal of Cell Biology*, *68*(22),

740–751.

Saheb, E., Trzyna, W., & Bush, J. (2014). Caspase-like proteins: *Acanthamoeba castellanii* metacaspase and *Dictyostelium discoideum* paracaspase, what are their functions? *Journal of Biosciences*, *39*, 909–916. https://doi.org/10.1007/s12038-014-9486-0

Saheb, E., Trzyna, W., Maringer, K., & Bush, J. (2015). Abnormalities of endocytosis, phagocytosis, and development process in *Dictyostelium* cells that over-express *Acanthamoeba castellanii* metacaspase protein. *Iranian Journal of Parasitology*, *10*(2), 213–229.

Sauvadet, A. L., Gobet, A., & Guillou, L. (2010). Comparative analysis between protist communities from the deep-sea pelagic ecosystem and specific deep hydrothermal habitats. *Environmental Microbiology*, *12*(11), 2946–2964. https://doi.org/10.1111/j.1462-2920.2010.02272.x

Sawyer, T. K., Nerad, T. A., & Visvesvara, G. S. (1992). *Acanthamoeba jacobsi sp. n.* (Protozoa: Acanthamoebidae) from sewage contaminated ocean sediments. *Journal of the Helminthological Society of Washington*, *59*(2), 223–226.

Scheid, P., Balczun, C., & Schaub, G. a. (2014). Some secrets are revealed: parasitic keratitis amoebae as vectors of the scarcely described pandoraviruses to humans. *Parasitology Research*, *113*(10), 3759–3764. https://doi.org/10.1007/s00436-014-4041-3

Schindler, C. A., & Schuhardt, V. T. (1964). Lysostaphin: A New Bacteriolytic Agent for the *Staphylococcus*. *Proceedings of the National Academy of Sciences of the United States of America*, *51*(1963), 414–421. https://doi.org/10.1073/pnas.51.3.414

Schmitz-Esser, S., Linka, N., Collingro, A., Beier, C. L., Neuhaus, H. E., Wagner, M., & Horn, M. (2004). ATP/ADP translocases: A common feature of obligate intracellular amoebal symbionts related to Chlamydiae and Rickettsiae. *Journal of Bacteriology*, *186*(3), 683–691. https://doi.org/10.1128/JB.186.3.683-691.2004

Schroeder-Diedrich, J. M., Fuerst, P. A., & Byers, T. J. (1998). Group-I introns with unusual sequences occur at three sites in nuclear 18S rRNA genes of *Acanthamoeba lenticulata*. *Current Genetics*, *34*(1), 71–78. https://doi.org/10.1007/s002940050368

Schroeder, J. M., Booton, G. C., Hay, J., Niszl, I. A., Seal, D. V., Markus, M. B., … Byers, T. J. (2001). Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of Acanthamoebae from humans with keratitis and from sewage sludge. *Journal of Clinical Microbiology*, *39*(5), 1903–1911. https://doi.org/10.1128/JCM.39.5.1903-1911.2001

Schulz, F., Lagkouvardos, I., Wascher, F., Aistleitner, K., Kostanjšek, R., & Horn, M. (2014). Life in an unusual intracellular niche: A bacterial symbiont infecting the nucleus of amoebae. *ISME Journal*, *8*(8), 1634–1644. https://doi.org/10.1038/ismej.2014.5

Schulz, F., Tyml, T., Pizzetti, I., Dyková, I., Fazi, S., Kostka, M., & Horn, M. (2015). Marine amoebae with cytoplasmic and perinuclear symbionts deeply branching in the Gammaproteobacteria. *Scientific Reports*, *5*(May), 1–10. https://doi.org/10.1038/srep13381

Schulze, I., & Jantzen, H. (1982). Coordinate regulation of synthesis of ribosomal proteins. *European Journal of Biochemistry*, *126*, 285–292.

Seal, D. V., Kirkness, C. M., Bennett, H. G. B., & Peterson, M. (1999). Population-based cohort study of microbial keratitis in Scotland: incidence and features. *Contact Lens and Anterior Eye*, *22*(2), 49–57. https://doi.org/10.1016/S1367-0484(99)80003-4

Shatilovich, A. V, Shmakova, L. A., Mylnikov, A. P., & Gilichinsky, D. A. (2009). Ancient protozoa isolated from permafrost. In R. Margesin (Ed.), *Permafrost soils, Soil Biology* (First, Vol. 16, pp. 97–115). Springer. https://doi.org/10.1007/978-3-540-69371-0

Shinnar, A. E., Butler, K. L., & Park, H. J. (2003). Cathelicidin family of antimicrobial peptides: proteolytic processing and protease resistance. *Bioorganic Chemistry*, *31*(6), 425–436. https://doi.org/10.1016/S0045-2068(03)00080-4

Shmakova, L. A., Fedorov-Davydov, D. G., & Rivkina, E. M. (2014). The amoeboid protists of cryogenic soils in the Kolyma Lowland. *Eurasian Soil Science*, *46*(12), 1211–1218.

https://doi.org/10.1134/S1064229314010116

Siddiqui, R., & Khan, N. A. (2012a). *Acanthamoeba* is an evolutionary ancestor of macrophages: a myth or reality? *Experimental Parasitology*, *130*(2), 95–97. https://doi.org/10.1016/j.exppara.2011.11.005

Siddiqui, R., & Khan, N. A. (2012b). Biology and pathogenesis of *Acanthamoeba*. *Parasites & Vectors*, *5*(1), 6. https://doi.org/10.1186/1756-3305-5-6

Siddiqui, R., & Khan, N. A. (2012c). War of the microbial worlds: who is the beneficiary in *Acanthamoeba*-bacterial interactions? *Experimental Parasitology*, *130*(4), 311–313. https://doi.org/10.1016/j.exppara.2012.01.021

Simmons, P. A., Tomlinson, A., & Seal, D. V. (1998). The role of *Pseudomonas aeruginosa* biofilm in the attachment of *Acanthamoeba* to four types of hydrogel contact lens materials. *Optometry and Vision Science*, *75*(12), 860–866. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9875990

Sims, G. P., Aitken, R., & Rogerson, A. (2002). Identification and phylogenetic analysis of morphologically similar naked amoebae using small subunit ribosomal RNA. *The Journal of Eukaryotic Microbiology*, *49*(6), 478–484. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12503684

Sims, G. P., Rogerson, A., & Aitken, R. (1999). Primary and secondary structure of the small-subunit ribosomal RNA of the naked, marine amoeba *Vannella anglica*: phylogenetic implications. *Journal of Molecular Evolution*, *48*(6), 740–749. https://doi.org/10.1007/PL00006518

Singh, B. N. (1948). Studies on giant amoeboid organisms. *Microbiology*, *2*(1), 89–96. https://doi.org/10.1099/00221287-2-1-89

Singh, B. N. (1950). A culture method for growing small free-living amoebae for the study of their nuclear division. *Nature*, *165*, 65–66.

Siritapetawee, J., Thumanu, K., Sojikul, P., & Thammasirirak, S. (2012). A novel serine protease with human fibrino(geno)lytic activities from *Artocarpus heterophyllus* latex. *Biochimica et Biophysica Acta*, *1824*(7), 907–912. https://doi.org/10.1016/j.bbapap.2012.05.002

Sissons, J., Alsam, S., Goldsworthy, G., Lightfoot, M., Jarroll, E. L., & Khan, N. A. (2006). Identification and properties of proteases from an *Acanthamoeba* isolate capable of producing granulomatous encephalitis. *BMC Microbiology*, *6*(42), 1–8. https://doi.org/10.1186/1471-2180-6-42

Sissons, J., Alsam, S., Jayasekera, S., & Khan, N. A. (2004). Ecto-ATPases of clinical and non-clinical isolates of *Acanthamoeba*. *Microbial Pathogenesis*, *37*(5), 231–239. https://doi.org/10.1016/j.micpath.2004.01.004

Slater, C. A., Sickel, J. Z., Visvesvara, G. S., Pabico, R. C., & Gaspari, A. A. (1994). Brief report: succesful treatment of disseminated *Acanthamoeba* infection in an immunocompromised patient. *The New England Journal of Medicine*, *331*(1), 85–87.

Smirnov, A., Nassonova, E., Berney, C., Fahrni, J., Bolivar, I., & Pawlowski, J. (2005). Molecular phylogeny and classification of the lobose amoebae. *Protist*, *156*(2), 129–142. https://doi.org/10.1016/j.protis.2005.06.002

Smirnov, A., Nassonova, E., Fahrni, J., & Pawlowski, J. (2009). *Rhizamoeba neglecta n. sp.* (Amoebozoa, Tubulinea) from the bottom sediments of freshwater Lake Leshevoe (Valamo Island, North-Western Russia), with notes on the phylogeny of the order Leptomyxida. *European Journal of Protistology*, *45*(4), 251–259. https://doi.org/10.1016/j.ejop.2009.04.002

Smirnov, A., Nassonova, E., Geisen, S., Bonkowski, M., Kudryavtsev, A., Berney, C., … Pawlowski, J. (2017). Phylogeny and systematics of leptomyxid amoebae (Amoebozoa, Tubulinea, Leptomyxida). *Protist*, *168*(2), 220–252. https://doi.org/10.1016/j.protis.2016.10.006

Smirnov, A., Nassonova, E., Holzmann, M., & Pawlowski, J. (2002). Morphological, ecological and molecular studies of *Vannella simplex* Wohlfarth-Bottermann 1960 (Lobosea,

Gymnamoebia), with a new diagnosis of this species. *Protist*, *153*, 367–377. https://doi.org/10.1078/14344610260450109

Smirnov, A. V. (2001). *Vannella ebro n. sp.* (Lobosea, Gymnamoebia), isolated from cyanobacterial mats in Spain. *European Journal of Protistology*, *37*(2), 147–153. https://doi.org/10.1078/0932-4739-00811

Smirnov, A. V., Chao, E., Nassonova, E. S., & Cavalier-Smith, T. (2011). A revised classification of naked lobose amoebae (Amoebozoa: Lobosa). *Protist*, *162*(4), 545–570. https://doi.org/10.1016/j.protis.2011.04.004

Smirnov, A. V., & Fenchel, T. (1996). *Vahlkampfia anaerobica n. sp.* and *Vannella peregrinia n. sp.* (Rhizopoda) anaerobic amoebae from a marine sediment. *Archiv Fur Protistenkunde*, *147*(2), 189–198. https://doi.org/10.1016/S0003-9365(96)80033-9

Smirnov, A. V., Nassonova, E. S., & Cavalier-Smith, T. (2008). Correct identification of species makes the amoebozoan rRNA tree congruent with morphology for the order Leptomyxida Page 1987; with description of *Acramoeba dendroida n. g., n. sp.*, originally misidentified as "*Gephyramoeba sp.'</i>*." *European Journal of Protistology*, *44*(1), 35–44. https://doi.org/10.1016/j.ejop.2007.08.001

Smirnov, A. V., Nassonova, E. S., Chao, E., & Cavalier-Smith, T. (2007). Phylogeny, evolution, and taxonomy of vannellid amoebae. *Protist*, *158*(3), 295–324. https://doi.org/10.1016/j.protis.2007.04.004

Smirnov, A. V, & Brown, S. (2000). First isolation of a cyst-forming *Vannella* species , from soil – *Vannella persistens n. sp.* (Gymnamoebia, Vannellidae). *Protistology*, *1*(3), 120–123.

Snider, C., Jayasinghe, S., Hristova, K., & White, S. H. (2009). MPEx: A tool for exploring membrane proteins. *Protein Science*, *18*(12), 2624–2628. https://doi.org/10.1002/pro.256

Sogin, M. L., & Gunderson, J. H. (1987). Structural diversity of eukaryotic small subunit ribosomal RNAs: evolutionary implications. *Annals of the New York Academy of Sciences*, *503*(1), 125–139. https://doi.org/10.1111/j.1749-6632.1987.tb40603.x

Song, S. M., Han, B. I., Moon, E. K., Lee, Y. R., Yu, H. S., Jha, B. K., … Hong, Y. (2012). Autophagy protein 16-mediated autophagy is required for the encystation of *Acanthamoeba castellanii*. *Molecular and Biochemical Parasitology*, *183*(2), 158–165. https://doi.org/10.1016/j.molbiopara.2012.02.013

Springer, N., Ludwig, W., Drożański, W., Amann, R., & Schleifer, K. H. (1992). The phylogenetic status of *Sarcobium lyticum*, an obligate intracellular bacterial parasite of small amoebae. *FEMS Microbiology Letters*, *96*(2–3), 199–202. Retrieved from http://dx.doi.org/10.1111/j.1574-6968.1992.tb05416.x

Sriram, R., Shoff, M., Booton, G., Fuerst, P., & Visvesvara, G. S. (2008). Survival of *Acanthamoeba cysts* after desiccation for more than 20 years. *Journal of Clinical Microbiology*, *46*(12), 4045–4048. https://doi.org/10.1128/JCM.01903-08

Steenbergen, J. N., Shuman, H. a, & Casadevall, A. (2001). *Cryptococcus neoformans* interactions with amoebae suggest an explanation for its virulence and intracellular pathogenic strategy in macrophages. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(26), 15245–15250. https://doi.org/10.1073/pnas.261418798

Stehr-Green, J. K., Bailey, T. M., & Visvesvara, G. S. (1989). The epidemiology of *Acanthamoeba* keratitis in the United States. *American Journal of Ophthalmology*, *107*(4), 331–336. https://doi.org/10.1016/0002-9394(89)90654-5

Steinert, M., Birkness, K., White, E., Fields, B., & Quinn, F. (1998). *Mycobacterium avium* bacilli grow saprozoically in coculture with *Acanthamoeba polyphaga* and survive within cyst walls. *Applied and Environmental Microbiology*, *64*(6), 2256–2261.

Stevens, a. R., & Pachler, P. F. (1973). RNA synthesis and turnover during density-inhibited growth and encystment of *Acanthamoeba castellanii*. *Journal of Cell Biology*, *57*(2), 525–537. https://doi.org/10.1083/jcb.57.2.525

Stothard, D. a, Schroeder-Diedrich, J. M., Awwad, M. H., Gast, R. J., Ledee, D. R., Rodriguez-

Zaragoza, S., … Byers, T. J. (1998). The evolutionary history of the genus *Acanthamoeba* and the identification of eight new 18S rRNA gene sequence types. *Journal of Eukaryotic Microbiology*, *45*(1), 45–54. https://doi.org/10.1016/j.ympev.2007.05.025

Strassmann, J. E., & Shu, L. (2017). Ancient bacteria–amoeba relationships and pathogenic animal bacteria. *PLoS Biology*, *15*(5), 1–8. https://doi.org/10.1371/journal.pbio.2002460

Suga, H., Chen, Z., de Mendoza, A., Sebé-Pedrós, A., Brown, M. W., Kramer, E., … Ruiz-Trillo, I. (2013). The *Capsaspora* genome reveals a complex unicellular prehistory of animals. *Nature Communications*, *4*, 1–9. https://doi.org/10.1038/ncomms3325

Tagai, C., Morita, S., Shiraishi, T., Miyaji, K., & Iwamuro, S. (2011). Antimicrobial properties of arginine- and lysine-rich histones and involvement of bacterial outer membrane protease T in their differential mode of actions. *Peptides*, *32*(10), 2003–2009. https://doi.org/10.1016/j.peptides.2011.09.005

Tice, A. K., Shadwick, L. L., Fiore-Donno, A. M., Geisen, S., Kang, S., Schuler, G. A., … Brown, M. W. (2016). Expansion of the molecular and morphological diversity of Acanthamoebidae (Centramoebida, Amoebozoa) and identification of a novel life cycle type within the group. *Biology Direct*, *11*(1), 1–21. https://doi.org/10.1186/s13062-016-0171-0

Titcomb, L., & Jamieson, C. (2013). Ophtalmic Infections. Guidelines for the management of *Acanthamoeba* Keratitis. *Sandwell and West Birmingham Hospitals NHS Trust*, *July*. Retrieved from http://bmec.swbh.nhs.uk/wp-content/uploads/2013/03/SWBH-Guidelines-for-the-Management-of-Acanthamoeba-Keratitis-July-2013.pdf

Titus, M. A., & Goodson, H. V. (2017). An evolutionary perspective on cell migration. *The Journal of Cell Biology*, *216*(6), 1509–1511. https://doi.org/10.1083/jcb.201704112

Todd, C., Reyes-Batlle, M., Valladares, B., Lindo, J., & Lorenzo-Morales, J. (2015). Vannellid species isolated from freshwater source in a park in Jamaica, West Indies. *Microbiology Insights*, *8*, 7–9. https://doi.org/10.4137/MBI.S30537

Tomlinson, G., & Jones, E. A. (1962). Isolation of cellulose from the cyst wall of a soil amoeba. *Biochimica et Biophysica Acta*, *63*(1), 194–200.

Turner, N. A., Russell, A. D., Furr, J. R., & Lloyd, D. (2000). Emergence of resistance to biocides during differentiation of *Acanthamoeba castellanii*. *The Journal of Antimicrobial Chemotherapy*, *46*(1), 27–34. https://doi.org/10.1093/jac/46.1.27

Turner, N. A., Russell, A. D., Furr, J. R., & Lloyd, D. (2004). Resistance, biguanide sorption and biguanide-induced pentose leakage during encystment of *Acanthamaoeba castellanii*. *Journal of Applied Microbiology*, *96*(6), 1287–1295. https://doi.org/10.1111/j.1365-2672.2004.02260.x

Tyndall, R. L., Lehman, E. S., Bowman, E. K., Milton, D. K., & Barbaree, J. M. (1995). Home humidifiers as a potential source of exposure to microbial pathogens, endotoxins, and allergens. *Indoor Air*, *5*(3), 171–178. https://doi.org/10.1111/j.1600-0668.1995.t01-1-00003.x

Upadhyay, M. K., Sharma, R., Pandey, A. K., & Rajak, R. C. (2005). An improved zymographic method for detection of amylolytic enzymes of fungi on polyacrylamide gels. *Mycologist*, *19*(4), 138–140. https://doi.org/10.1017/S0269915XO5004015

van Dyk, J. S., Sakka, M., Sakka, K., & Pletschke, B. I. (2010). Identification of endoglucanases, xylanases, pectinases and mannanases in the multi-enzyme complex of *Bacillus licheniformis* SVD1. *Enzyme and Microbial Technology*, *47*(3), 112–118. https://doi.org/10.1016/j.enzmictec.2010.05.004

Visvesvara, G. S. (1991). Classification of *Acanthamoeba*. *Reviews of Infectious Diseases*, *13*, S369–S372. https://doi.org/10.1093/clind/13.Supplement_5.S369

Visvesvara, G. S. (2010). Amebic meningoencephalitides and keratitis: challenges in diagnosis and treatment*. *Current Opinion in Infectious Diseases*, *23*(6), 590–594. https://doi.org/10.1097/QCO.0b013e32833ed78b

Visvesvara, G. S., Booton, G. C., Kelley, D. J., Fuerst, P., Sriram, R., Finkelstein, A., & Garner, M. M. (2007). In vitro culture, serologic and molecular analysis of *Acanthamoeba* isolated

from the liver of a keel-billed toucan (*Ramphastos sulfuratus*). *Veterinary Parasitology*, *143*(1), 74–78. https://doi.org/10.1016/j.vetpar.2006.08.010

Visvesvara, G. S., Martinez, A. J., Schuster, F. L., Leitch, G. J., Wallace, S. V., Sawyer, T. K., & Anderson, M. (1990). Leptomyxid ameba, a new agent of amebic meningoencephalitis in humans and animals. *Journal of Clinical Microbiology*, *28*(12), 2750–2756.

Visvesvara, G. S., Roy, S. L., & Maguire, J. H. (2011). Pathogenic and opportunistic free-living amoebae: *Acanthamoeba spp., Balamuthia mandrillaris, Naegleria fowleri*, and *Sappinia pedata*. In R. L. Guerrant, D. H. Walker, & P. F. Weller (Eds.), *Tropical Infectious Diseases: Principles, Pathogens and Practice* (Thrid Edit, pp. 707–713). Elsevier Health Sciences. https://doi.org/10.1016/B978-0-7020-3935-5.00101-4

Visvesvara, G. S., Schuster, F. L., & Martinez, A. J. (1993). *Balamuthia mandrillaris, N. G., N. Sp.,* agent of amebic meningoencephalitis in humans and other animals. *Journal of Eukaryotic Microbiology*, *40*(4), 504–514.

Volkonsky, M. (1931). “Hartmannella castellanii” *Douglas et classification des Hartmannelles (“Hartmanellinae” nov. subfam.," Acanthamoeba" nov. gen.," Glaeseria" nov. gen.)*. H. Le Soudier.

Walia, R., Montoya, J. G., Visvesvera, G. S., Booton, G. C., & Doyle, R. L. (2007). A case of successful treatment of cutaneous *Acanthamoeba* infection in a lung transplant recipient. *Transplant Infectious Disease*, *9*(1), 51–54. https://doi.org/10.1111/j.1399-3062.2006.00159.x

Walochnik, J., & Mulec, J. (2009). Free-living amoebae in carbonate precipitating microhabitats of karst caves and a new vahlkampfiid amoeba, *Allovahlkampfia spelaea gen. nov., sp. nov*. *Acta Protozoologica*, *48*(1), 25–33. https://doi.org/10.1111/j.1755-3768.2011.02269.x

Walochnik, J., Picher, O., Aspöck, C., Ullmann, M., Sommer, R., & Aspöck, H. (1999). Interactions of “Limax amoebae” and gram-negative bacteria: experimental studies and review of current problems. *The Tokai Journal of Experimental and Clinical Medicine*, *23*, 273–278.

Watts, D. J., & Ashworth, J. M. (1970). Growth of myxameobae of the cellular slime mould *Dictyostelium discoideum* in axenic culture. *The Biochemical Journal*, *119*(2), 171–174. https://doi.org/10.1042/BJ1190171

Webster, D., Umar, I., Kolyvas, G., Bilbao, J., Guiot, M. C., Duplisea, K., … Visvesvara, G. S. (2012). Case report: treatment of granulomatous amoebic encephalitis with voriconazole and miltefosine in an immunocompetent soldier. *American Journal of Tropical Medicine and Hygiene*, *87*(4), 715–718. https://doi.org/10.4269/ajtmh.2012.12-0100

Weekers, P. H., Gast, R. J., Fuerst, P. a, & Byers, T. J. (1994). Sequence variations in small-subunit ribosomal RNAs of *Hartmannella vermiformis* and their phylogenetic implications. *Molecular Biology and Evolution*, *11*(4), 684–690.

Weekers, P. H. H., Bodelier, P. L. E., Wijen, J. P. H., & Vogels, G. D. (1993). Effects of grazing by the free-living soil amoebae *Acanthamoeba castellanii, Acanthamoeba polyphaga*, and *Hartmanella vermiformis* on various bacteria. *Applied and Environmental Microbiology*, *59*(7), 2317–2319.

Weekers, P. H. H., & Drift, C. Van Der. (1993). Nitrogen metabolizing enzyme activities in the free-living soil amoebae *Acanthamoeba castellanii, Acanthamoeba polyphaga* and *Hartmannella vermiformis*. *Journal of Eukaryotic Microbiology*, *40*(3), 251–254. https://doi.org/10.1111/j.1550-7408.1993.tb04912.x

Weisman, R. A., & Korn, E. D. (1967). Phagocytosis of latex beads by *Acanthamoeba*. I. Biochemical properties. *Biochemistry*, *6*(2), 485–497. https://doi.org/10.1021/bi00854a017

Weisman, R. A., & Shaw, G. B. (1976). Differentiation in *Acanthamoeba castelanii*. *Annual Review of Microbiology*, *30*, 189–219.

Weisman, R. A., Spiegel, R. S., & Mccauley, J. G. (1969). Differentiation in *Acanthamoeba*: Glycogen levels and glycogen sythetase activity during encystment. *Biochimica et Biophysica Acta*, *201*, 45–53.

Wheat, W. H., Casali, A. L., Thomas, V., Spencer, J. S., Lahiri, R., Williams, D. L., … Jackson, M. (2014). Long-term survival and virulence of *Mycobacterium leprae* in amoebal cysts. *PLoS Neglected Tropical Diseases*, *8*(12). https://doi.org/10.1371/journal.pntd.0003405

Willis, J. D., Klingeman, W. E., Oppert, C., Oppert, B., & Jurat-Fuentes, J. L. (2010). Characterization of cellulolytic activity from digestive fluids of *Dissosteira carolina* (Orthoptera: Acrididae). *Comparative Biochemistry and Physiology*, *157*(3), 267–272. https://doi.org/10.1016/j.cbpb.2010.06.012

Woese, C. R. (1987). Bacterial evolution. *Microbiological Reviews*, *51*(2), 221–271. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2439888%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC373105

Woods, L. E., Cole, C. V., Elliott, E. T., Anderson, R. V., & Coleman, D. C. (1982). Nitrogen transformations in soil as affected by bacterial-microfaunal interactions. *Soil Biology and Biochemistry*, *14*(2), 93–98. https://doi.org/10.1016/0038-0717(82)90050-5

Yu, H. S., Choi, K. H., Kim, H. K., Kong, H. H., & Chung, D. I. (2001). Genetic analyses of *Acanthamoeba* isolates from contact lens storage cases of students in Seoul, Korea. *Korean Journal of Parasitology*, *39*(2), 161–170.

Zauberman, N., Mutsafi, Y., Halevy, D. Ben, Shimoni, E., Klein, E., Xiao, C., … Minsky, A. (2008). Distinct DNA exit and packaging portals in the virus *Acanthamoeba polyphaga mimivirus*. *PLoS Biology*, *6*(5), 1104–1114. https://doi.org/10.1371/journal.pbio.0060114

Zhou, X., Elmose, J., & Call, D. R. (2007). Interactions between the environmental pathogen *Listeria monocytogenes* and a free-living protozoan (*Acanthamoeba castellanii*). *Environmental Microbiology*, *9*(4), 913–922. https://doi.org/10.1111/j.1462-2920.2006.01213.x